CHAPTER 3

EXPERIMENTAL MODEL FOR CIGARETTE-SMOKE -INHALATION STUDIES

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A. Introduction

Attempts to approximate the smoking habit of humans have involved forcing or training animals to inhale air through a burning cigarette (Jarvick 1967; Albert *et al* 1969; Auerbach *et al* 1970; Stuart, Willard and Howard 1970). This has been accomplished with the aid of masks and tracheotomies (Albert *et al* 1969; Auerbach *et al* 1970a; Stuart *et al* 1970). These active inhalation procedures are suitable for large animals such as dogs (Auerbach *et al* 1970a) and donkeys (Albert *et al* 1969) but are time consuming, expensive and unsuitable for small laboratory animals (Hoffmann and Wynder 1970).

Several passive inhalation techniques have been developed to force small animals to inhale cigarette smoke generated in an exposure chamber (Hoffmann and Wynder 1970). Since particulate aggregation as well as physical and chemical aging occurs in cigarette smoke it was necessary to develop machines which could present animals with a fresh cigarette smoke (Hoffmann and Wynder 1970). A number of machines designed to produce fresh mixtures of cigarette smoke and air have been developed and used to produce pathological changes in small rodents (Harris and Negroni 1967; Dontenwill 1970; Leuchtenberger and Leuchtenberger 1970a; Dontenwill *et al* 1973).

In the experiments described in this thesis, a Hamburg II smoking apparatus (Dontenwill 1970), which was commercially available (Heinr Borgwaldt, West Germany), was used to expose mice to cigarette smoke. The machine was set to present puffs of fresh cigarette smoke in a 1:7 smoke:air ratio, with a constant rhythm of smoke flow. On five consecutive days of each week mice were exposed to the puffs from 30 cigarettes (7 minutes continuous smoke exposure per day). The smoke exposure conditions were intended to subject the mice to acceptable concentrations of cigarette smoke while avoiding acute toxic effects and permitting the mice to live an acceptable life span (Dontenwill 1970).

It was, however, considered necessary not only to standardize the exposure conditions but also to submit the mice to a battery of tests which could assist in determining the amount of smoke inhalation and the sensitivity of the animals to the toxic effects of cigarette smoke. Therefore the measurements of blood carboxyhaemoglobin, body weights, organ weights, food consumption

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and haematological values of mice exposed to cigarette smoke which are described in this chapter were determined to help define the murine model used for the immunological studies.

B. Blood carboxyhaemoglobin

The carboxyhaemoglobin concentration (%HbCO) in the peripheral blood of BALB/c mice was measured after 1/2, 1, 2 and 3 rounds of exposure to cigarette smoke (Fig. 4) (1 round of cigarettes is the exposure of the mice to 30 cigarettes over a standardised time of 7 minutes). The carboxyhaemoglobin concentrations were also measured for up to an hour after the third round of cigarettes (Fig. 4). The mice developed high levels of carboxyhaemoglobin (30.6% of the haemoglobin) but these rapidly declined after discontinuing the exposure to cigarette smoke. The concentration of carboxyhaemoglobin was measured in 6 strains of mice after 1 round of cigarette smoke exposure (Table 1). The carboxyhaemoglobin levels of C57Black C3H and DK Black mice were significantly higher than the levels in BALB/c mice ($p<0.05$). The C57Black mice also had a significantly higher concentration than Simpson mice ($p<0.05$).

C. Toxicity of cigarette smoking

To measure the acute toxic effects of the inhalation of cigarette smoke, C57Black mice were continuously exposed to cigarette smoke. Generally 8 rounds of cigarette-smoke exposure was lethal for 50% of the mice (56 minutes of continuous exposure to cigarette smoke). No controlled toxicity study for chronic exposure was performed but few mice died after periods of exposure up to 42 weeks. Generally all the mice appeared to be in good condition after chronic exposure to cigarette smoke.

D. Body-weight and diet

The body weight and diet of 8 week old C57Black mice were measured after periods up to 80 days of exposure to cigarette smoke (Table 2). The mice exposed to cigarette smoke lost some weight and maintained a lower body-weight than age matched controls. Mice

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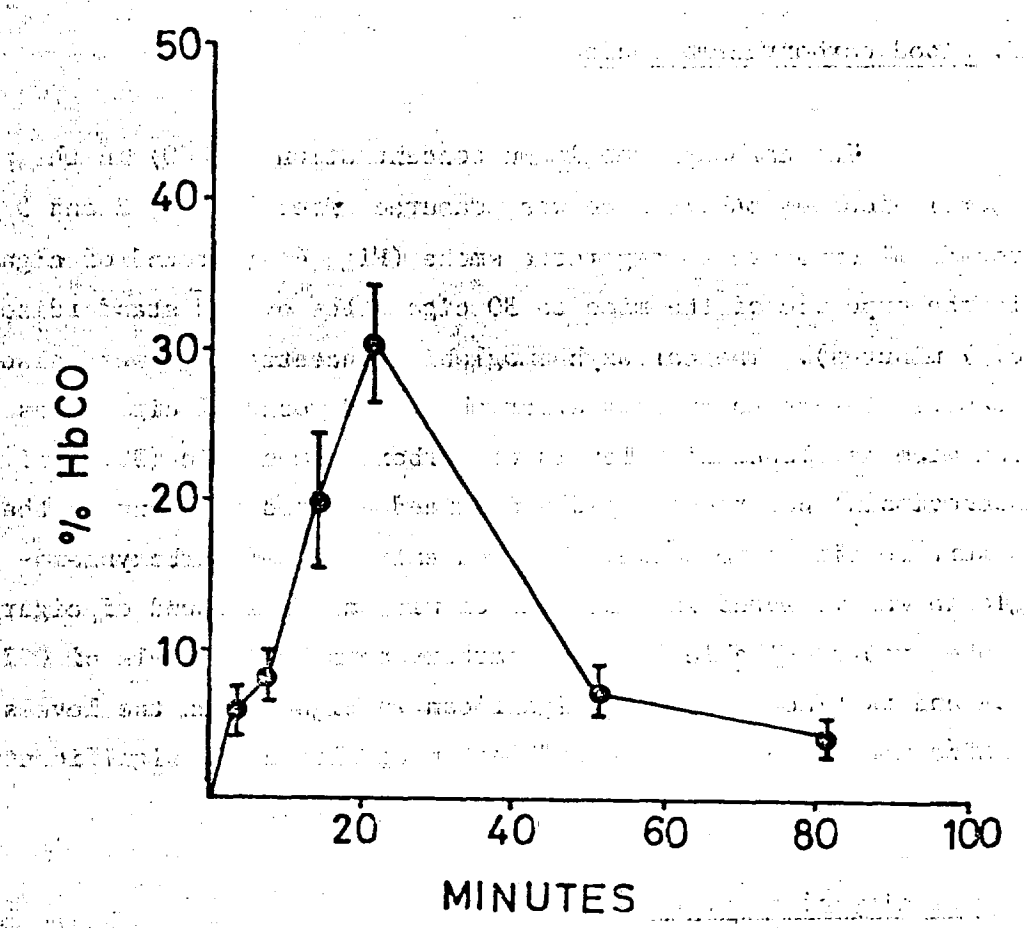

FIGURE 4 Blood carboxyhaemoglobin levels in BALB/c mice exposed to cigarette smoke. Mice were exposed for 1/2 (3.5 minutes), 1 (7 minutes), 2 (14 minutes) and 3 rounds of cigarettes (21 minutes), in a Hamburg II smoking machine. Each result is the mean ± S.E. of the values from 5 mice: † smoke-exposure discontinued.

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TABLE 1 Blood carboxyhaemoglobin in six strains of mice after inhalation of cigarette smoke

Mouse strain	% Carboxyhaemoglobin (mean ± S.E.)
1. BALB/c	8.7 ± 2.1
2. Simpson	12.5 ± 3.3
3. DK Ginger	17.4 ± 2.4
4. DK Black	20.0 ± 3.1
5. C3H	21.7 ± 2.1
6. C57Black	22.7 ± 2.8

The significance of differences between the % carboxyhaemoblobin was examined by Student's t test. Strains 4 and 5 were significantly different from strain 1 ($p<0.05$). Strain 6 was significantly different from strain 1 and 2 ($p<0.05$).

TABLE 2 Body weight and diet of C57Black mice exposed to cigarette smoke

Days smoke exposure	Group	Body-weight (g)	Food consumption (g/mouse/day) (moist wt.)(2)
0	exposed	18.4 ± 0.3(1)	
	control	18.3 ± 0.06	
10	exposed	17.7 ± 0.3***	2.0 ± 0.01**
	control	19.4 ± 0.2	2.7 ± 0.1
24	exposed	17.9 ± 0.2***	2.0 ± 0.03*
	control	19.7 ± 0.2	2.4 ± 0.03
44	exposed	18.5 ± 0.3***	
	control	20.5 ± 0.2	
80	exposed	18.6 ± 0.3***	1.9 ± 0
	control	21.4 ± 0.3	2.0 ± 0.1

(1) Each result is the mean ± S.E. of the values from 20 mice.
(2) The food consumption of cages of mice was measured over a period of one week. Each result is the mean ± S.E. of the values from 3 cages.

The significance of the differences between the values from control mice and mice exposed to cigarette smoke was determined by Student's t test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

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exposed to cigarette smoke also had a 7.6% lower body weight than the controls when measured after 28 weeks cigarette-smoke exposure. The weight of mouse food-cubes consumed by groups of mice exposed to cigarette smoke and control mice during one week was measured after 10, 24 and 80 days of exposure to cigarette smoke (Table 2). The mice exposed to cigarette smoke had a decreased food intake.

E. Organ weights

The weights of the lungs, spleens, livers and kidneys of C57Black female mice exposed to cigarette smoke for 18 and 34 weeks were measured (Tables 3,4). No significant differences were found between the weights of organs from cigarette-smoke exposed or age-matched-control mice.

F. Haematology

The haematological data of groups of 10 - 20 mice were determined after 8, 20, 24 and 35 weeks cigarette-smoke exposure (Tables 5, 6, 7, 8). The changes occurring in leucocyte and erythrocyte counts, haemoglobin concentration, packed-cell volumes and mean cell volumes are summarised in Table 9. The erythrocyte counts and concentrations of haemoglobin of mice exposed to cigarette smoke showed a transient increase but after 35 weeks exposure fell to below the levels of age-matched-controls. The leucocyte count was depressed when measured after only 8 weeks inhalation of cigarette smoke. No differences in the differential leucocyte counts of control mice and mice exposed to cigarette smoke were found.

G. Discussion

Experimentation into the effect of cigarette smoke on laboratory animals has depended on the development of machines which can force animals to inhale a suitable amount of fresh cigarette-smoke (Leuchtenberger and Leuchtenberger 1968; Dontenwill 1970; Hoffmann and Wynder 1970). The Hamburg II inhalation apparatus (Dontenwill 1970) used in the experiments of this programme has been described as one of the most advanced designs of inhalation chambers

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TABLE 3 Organ weights in C57Black mice exposed to cigarette smoke for 18 weeks

Organ	Organ weight (g moist weight)* Smoke-exposed	Control
Lungs	0.19 ± 0.01	0.17 ± 0.02
Liver	0.89 ± 0.03	0.93 ± 0.05
Kidney	0.25 ± 0.02	0.24 ± 0.02
Spleen	0.102 ± 0.01	0.094 ± 0.02

* Each result is the mean ± S.E. of values from 6 mice. No significant difference was found between the organs of test and control mice as judged by Student's t test.

TABLE 4 Organ weights in C57Black mice exposed to cigarette smoke for 34 weeks

Organ	Organ weight (g moist weight)* Smoke-exposed	Control
Lungs	0.17 ± 0.02	0.19 ± 0.01
Liver	0.93 ± 0.08	1.07 ± 0.04
Kidney	0.25 ± 0.02	0.25 ± 0.02
Spleen	0.087 ± 0.02	0.109 ± 0.02

* Each result is the mean ± S.E. of values from 6 mice. No significant difference was found between the organs of cigarette smoke exposed and control mice as judged by Student's t test.

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TABLE 5 Haematological data of C57Black female mice exposed to cigarette smoke for 8 weeks

Specification		Exposed	Control
Erythrocyte count (N^o/mm^3)x10^{-6}		9.8 ± 0.07 (1)	9.7 ± 0.07
Haemoglobin concentration (g/100ml)		15.0 ± 0.11	14.9 ± 0.09
Packed cell volume (%)		44.2 ± 0.33	45.0 ± 0.29
Mean cell volume (μ^3)		44.9 ± 0.04	45.2 ± 0.02
Mean cell haemoglobin (µµg)		15.6 ± 0.04	15.6 ± 0.03
Mean cell haemoglobin concentration (%)		33.1 ± 0.09	32.9 ± 0.09
Leucocyte count (N^o/mm^3)x10^{-3}	***	7.8 ± 0.37	10.6 ± 0.45
Differential leucocyte count			
Neutrophils (%)		10.7 ± 0.90	11.9 ± 1.1
Lymphocytes (%)		85.4 ± 1.1	84.6 ± 0.90
Monocytes (%)		4.1 ± 0.45	3.7 ± 0.56

(1) Each result is the mean ± S.E. of the values from 10 mice. The significance of the difference between smoke-exposed and control animals was examined with Student's t test: * $p<0.05$, *** $p<0.001$.

TABLE 6 Haematological data of C57Black female mice exposed to cigarette smoke for 20 weeks

Specification		Exposed	Control
Erythrocyte count (N^o/mm^3x10^{-6})		9.9 ± 0.09 (1)	9.8 ± 0.09
Haemoglobin concentration (g/100ml)		14.4 ± 0.13	14.2 ± 0.16
Packed cell volume (%)	*	45.9 ± 0.36	44.3 ± 0.40
Mean cell volume (μ^3)	*	45.4 ± 0.25	44.5 ± 0.11
Mean Cell haemoglobin (µµg)		14.8 ± 0.16	14.6 ± 0.07
Mean cell haemoglobin concentration (%)		31.2 ± 0.16	31.9 ± 0.13
Leucocyte count (N^o/mm^3x10^{-3})	*	4.8 ± 0.31	6.7 ± 0.36
Differential leucocyte count			
Neutrophils (%)		13.5 ± 1.6	12.6 ± 1.1
Lymphocytes (%)		85.1 ± 1.5	85.9 ± 1.2
Monocytes (%)		1.4 ± 0.31	1.5 ± 0.25

(1) Each result is the mean ± S.E. of the values from 20 mice. The significance of the difference between the smoke-exposed and control groups was tested by Student's t test: * $p<0.05$.

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TABLE 7 Haematological data of C57Black male mice exposed to cigarette smoke for 23 weeks

Specification		Exposed	Control
Erythrocyte count ($N^o mm^3 x10^{-6}$)	***	9.7 ± 0.09	9.3 ± 0.07
Haemoglobin concentration (g/100ml)	***	14.3 ± 0.09	13.5 ± 0.07
Packed cell volume (%)	***	45.1 ± 0.38	42.5 ± 0.25
Mean cell volume (μ^3)	**	44.4 ± 0.11	43.9 ± 0.09
Mean cell haemoglobin (µµg)		14.8 ± 0.03	14.7 ± 0.04
Mean cell haemoglobin concentration (%)		31.4 ± 0.09	31.4 ± 0.09
Leucocyte count ($N^o/mm^3 x10^{-3}$)	**	7.1 ± 0.38	8.9 ± 0.40
Differential leucocyte count			
Neutrophils (%)		12.4 ± 1.1	14.6 ± 1.1
Lymphocytes (%)		84.9 ± 1.0	82.1 ± 1.2
Monocytes (%)		2.8 ± 0.34	3.2 ± 0.42

Each result is the mean ± S.E. of the values of 20 mice. The significance of the difference between the smoke-exposed group and the controls was examined by Student's t test: ** $p<0.01$, *** $p<0.001$.

TABLE 8 Haematological data of C57Black male mice exposed to cigarette smoke for 35 weeks

Specification		Exposed	Control
Erythrocyte count ($N^o/mm^3 x10^{-6}$)	**	9.5 ± 0.11	10.1 ± 0.19
Haemoglobin concentration (g/100ml)	*	14.1 ± 0.16	14.8 ± 0.28
Packed cell volume (%)	*	43.8 ± 0.56	46.2 ± 0.85
Mean cell volume (μ^3)		44.6 ± 0.20	44.6 ± 0.16
Mean cell haemoglobin (µµg)	**	14.9 ± 0.07	14.7 ± 0.02
Mean cell haemoglobin concentration (%)		32.2 ± 0.16	32.0 ± 0.09
Leucocyte count ($N^o/mm^3 x10^{-3}$)	***	6.4 ± 0.36	9.0 ± 0.52
Differential leucocyte count			
Neutrophils (%)		15.2 ± 1.2	13.0 ± 0.94
Lymphocytes (%)		81.6 ± 1.6	84.4 ± 1.2
Monocytes (%)		3.2 ± 0.47	2.6 ± 0.45

Each result is the mean ± S.E. of the values from 20 mice. The significance of the difference between the smoke-exposed and control mice was examined by Student's t test:
* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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TABLE 9 Haematological data of C57Black mice exposed to cigarette smoke expressed as a percent of the values of control mice

	Weeks exposure to smoke			
	8 week	20 week	23 week	35 week
Leucocyte count	*** 73.6	* 71.6	** 80.0	*** 71.1
Erythrocyte count	101.0	101.0	*** 104.0	** 94.0
Haemoglobin concentration	100.7	* 101.4	*** 106.0	* 95.0
Packed cell volume	98.2	* 103.6	** 106.1	* 94.8
Mean cell volume	99.0	* 102.0	101.0	100.0

The values shown are derived from data presented in Tables 5 - 8. Each value is the mean of the values from a group of mice exposed to cigarette smoke shown as a percentage of the corresponding mean control value. The significance of the differences between the smoked mice and controls was determined by Student's t test.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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(Hoffmann and Wynder 1970). There are, however, obvious reasons for expecting that the experimental model used does not duplicate the human cigarette-smoking habit. Passively inhaling the smoke produced from 30 cigarettes for 7 consecutive minutes each day is different from the human habit, where cigarettes are typically smoked intermittantly during the day. Because the diameters and lengths of the airways, as well as the respiration rates of mice, are different from humans the distribution and depositions of the components of cigarette smoke in humans and mice are probably different (Dontenwill 1970). In any case the differences which distinguish mice from men also probably ensure that they react differently with the components of cigarette smoke.

Experimental and epidemiological studies of the effect of cigarette-smoke inhalation on humans are limited by factors such as genetic and cultural variations of humans, their different smoking habits and the inconvenience and discomfort produced by some experimental procedures. In addition to the availability of inbred animals, the use of experimental animals can also present the opportunity to study phenomena which are isolated or pronounced in animals but not in humans. Animals can be chosen for an experimental model because their size, availability and longevity may assist experimentation. As cigarette-smoke probably has different effects on different animals the use of several different animal models may be required to investigate the various aspects of cigarette smoking. This argument also applies to the different methods used to expose animals to cigarette smoke. Variation in the effect of cigarette smoke on different animals is illustrated by the large differences in the carboxyhaemoglobin levels produced in different strains of mice by cigarette-smoke inhalation (Table 1). Similar results have been reported (Dontenwill 1970). A standard regime of cigarette-smoke exposure has also been found to produce increased incidence of neoplasia in one strain of mice and cardiovascular disease in another (Leuchtenberger and Leuchtenberger 1974). While these results emphasise the difficulty in obtaining a comprehensive experimental-animal model for studying the effect of cigarette-smoke inhalation, they also indicate that the effect of cigarette smoking on different humans may be quite varied. This may be important in determining types of people who are at risk of developing the

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diseases associated with cigarette smoking.

The level of over 20% carboxyhaemoglobin which was reached by C57Black mice after one exposure to cigarette smoke shows that the mice were receiving at least an adequate exposure to the vapour phase of cigarette smoke. This level is not often achieved by humans and can produce neurological symptoms (Peterson and Stewart 1970; World Health Organization 1972). The study on carboxyhaemoglobin as well as body-weight, diet and toxicity produced results very similar to those of Dontenwill (1970), Reckzeh and Dontenwill (1970) and Dontenwill (1973). The mice exposed to cigarette smoke had depressed body-weights, associated with a decreased intake of food, but no differences were found between weights of organs from control mice and from mice exposed to cigarette smoke. However the errors associated with the estimation of the mean body-weights were less than the errors of estimating the mean organ-weights. Exposure to cigarette smoke also produced an increase in the erythrocyte counts and haemoblobin concentrations of mice, similar to the increase of erythrocyte count found in hamsters exposed to cigarette smoke (Reckzeh and Dontenwill 1970; Dontenwill *et al* 1973). However, prolonged exposure to cigarette smoke (35 weeks) decreased the erythrocyte counts and haemoglobin concentrations of the mice. Hamsters, exposed to cigarette smoke for a comparable period did show reductions in erythrocyte and haemoglobin levels (Dontenwill *et al* 1973). Although mice exposed to cigarette smoke eventually had a lower erythrocyte counts and haemoglobin concentrations than age-matched-control mice the values were well within the normal range. The most marked change in the haematology of the mice exposed to cigarette smoke was the large decrease in the number of peripheral-blood leucocytes. Reckzeh and Dontenwill (1970) and Dontenwill *et al* (1973) reported that leucocyte counts of hamsters were not significantly decreased by cigarette-smoke inhalation. Their results showed that the animals exposed to cigarette smoke consistantly had lower leucocyte counts than the controls and the counts became close to control levels after discontinuing the exposure to cigarette smoke (Reckzeh and Dontenwill 1970). In contrast to mice, cigarette smokers have an elevated number of peripheral-blood leucocytes (Corre *et al* 1971; Friedman *et al* 1973) but there is evidence indicating that this may be caused by the increased incidence

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of respiratory infection in cigarette smokers (Friedman *et al* 1973).

Although the carboxyhaemoglobin of mice exposed to cigarette smoke was high, the daily 7 minute exposures to cigarette smoke were considerably below acute lethal doses. This daily exposure could produce demonstrable effects on the mice, such as changes in body weight, but the mice were apparently healthy and could be maintained in experiments for prolonged periods.

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Taking into account the similarities between these observations and those of other studies, as well as the large body of evidence implicating cigarette smoking as the cause of a wide variety of diseases (Royal College of Physicians 1971; United States Public Health Service 1971), it was concluded that the experimental system was suitable for studying the effect of inhalation of cigarette smoke on the immune system of mice.

Question

rationale for using mouse inhal. system only, hence

each model was evaluated against the similarity of effects in mice

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CHAPTER 4

IMMUNOLOGICAL STUDIES

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SECTION 1 HUMORAL IMMUNITY

A. Introduction

The initial experiments described in this section are concerned with immune responses of normal mice. In order to study the effects of cigarette smoke on the immune responses of mice, it was considered important to examine the local and systemic immune responses to immunogens introduced into the respiratory system. Accordingly, the ability of intratracheal inoculations of sheep erythrocytes (SRBC) to elicit an antibody-forming-cell response in the lungs, the lymph nodes draining the respiratory system, the spleen and blood of mice was determined. The Cunningham and Szensberg modification of the Jerne plaque technique (Cunningham and Szensberg 1968) was employed to study the antibody-forming-cell responses. Both direct and indirect plaque-forming cells (pfc) were examined which can generally be equated with IgM antibody-producing cells and IgG or IgA antibody-producing cells respectively (Sterzl and Riha 1965). The nature of the responses was examined further by determining the effect of combinations of intravenous and intratracheal inoculations.

The humoral immune responses, of mice chronically exposed to cigarette smoke, to intratracheal inoculations of SRBC as well as to intraperitoneal inoculations of SRBC were then studied. As well as the responses to SRBC, an immunogen considered to require thymus-dependent lymphocytes (T-cells) (Davies, Leuchars Wallis and Doenhoff 1971; Playfair 1971), the antibody response to a T-cell independent immunogen, polyvinylpyrrolidone (PVP) (Andersson and Blomgren 1971), was also studied.

B. Local and systemic immune response of mice after intratracheal and intravenous inoculations of SRBC

In this section the antibody-forming-cell response, as estimated by the numbers of haemolytic-plaque-forming cells (pfc), occurring in normal mice after intratracheal and intravenous inoculations of sheep erythrocytes is described. The pfc in cell suspensions prepared from the lungs, spleen, blood and mediastinal and cervical lymph nodes were examined. The reproducibility of the

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intratracheal inoculation can be judged by the results of experiments described in Chapter 4 section 3.B where the technique has been used to deliver radioactive bacteria to the lungs.

(a) Plaque-forming cells in non-immunized mice

Plaque-forming cells were not detected in the lungs, blood or pools of mediastinal and cervical lymph nodes of 5 normal C57Black mice. In the spleens 1.6 ± 0.2 direct pfc/10^6 (mean ± S.E.) leucocytes were detected.

(b) Lymph nodes responding to intratracheal inoculations

Groups of five C57Black mice were inoculated intratracheally with 10^8 SRBC then examined 7 or 9 days later. The mediastinal, cervical and axillary lymph nodes were removed and cell suspensions prepared separately and suspended in a final volume of 0.1ml of growth medium. They were then tested for direct and indirect pfc (Table 10). The largest and most consistent response was found in the mediastinal lymph nodes when considered on a per organ or per 10^6 leucocytes basis. The cervical lymph nodes also made a large contribution to the response. However axillary lymph nodes had little or no response to intratracheal inoculations.

(c) Response in the spleen, lungs, blood and cervical and mediastinal lymph nodes to intravenous and intratracheal inoculations of SRBC

C57Black mice were inoculated intravenously or intratracheally with SRBC and then on day 12, mice from each group were challenged either intravenously or intratracheally. Groups of five mice from the four inoculation regimes were removed on days 3, 6, 7, 12, 15, 18, 21 and 24 after the first inoculation. They were examined for direct and indirect pfc in the lungs, blood, spleen and the pool of cervical and mediastinal lymph nodes. In order to test the correlation of pfc in organs and pfc freely circulating in the blood the results of direct and indirect pfc were regarded as separate values in the same set and the product-moment correlation coefficient (r) was calculated (Campbell 1967). Each value in the results (Figs. 5 - 8) represents the mean ± S.E. of values from 5 mice.

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TABLE 10 Plaque-forming cells in lymph nodes after an intratracheal inoculation of SRBC

	Days after inoculation	Lymph Nodes					
		Mediastinal		Cervical		Axillary	
		Direct	Indirect	Direct	Indirect	Direct	Indirect
pfc/organ	7	32 ± 22 (1)	31 ± 22	23 ± 19	7 ± 2	0	0
	9	20 ± 14	35 ± 33	2 ± 1	14 ± 6	0.5 ± 0.5	0
pfc/10^6 leucocytes	7	89 ± 53	82 ± 58	12 ± 7	4 ± 1	0	0
	9	43 ± 15	57 ± 42	2.4± 1	9.6± 2	1.9 ± 1.5	0

(1) Each value is the mean ± S.E. of the values from five mice.

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(i) Response of mice to intravenous inoculations (Fig. 5)

After the intravenous inoculation of 10^8 SRBC a large response occurred in the spleen with primarily direct pfc detected on day 3 followed by a predominantly indirect pfc response reaching a maximum on day 6. A small number of direct and indirect pfc was found in the blood on day 6 but few pfc were found in either the lymph nodes or the lungs. However, after a second intravenous inoculation, significant numbers of pfc were found in all organs examined. An anamnestic response occurred in the spleen for both direct and indirect pfc showing a maximum reaction after 3 days. The blood and the lungs showed the greatest number of pfc 6 days after the second inoculation. The blood had more pfc/10^6 leucocytes than the lungs, both having approximately equal proportions of direct and indirect pfc. The response shown in the lungs and blood was highly correlated ($r = 0.76$, $p<0.001$) (Campbell 1967). A response consisting primarily of indirect pfc was found in the lymph nodes, also being maximal at 6 days after boosting. However no relationship between the pfc found in the blood and lymph nodes was obvious ($r = 0.086$).

(ii) Response of mice to intratracheal inoculations (Fig. 6)

After intratracheal inoculation the spleen was still the organ containing the largest total number of pfc. However when considered per 10^6 leucocytes, the lymph nodes and the lungs had the most intense response. These organs had a transient response of both direct and indirect pfc being maximal on day 6. A small response of direct pfc was found in the blood on day 6.

Following a second intratracheal inoculation, an anamnestic response was found in the lungs and the lymph nodes showing an increased and prolonged indirect pfc involvement. No pfc appeared in the blood. The spleen showed a secondary response consisting primarily of indirect pfc, and still contained the largest number of pfc.

(iii) Intravenous inoculation of mice sensitized by intratracheal inoculation (Fig. 7)

The largest number of pfc found in the lungs and lymph nodes, after the inoculation regimes tested, was obtained when the animals were first inoculated intratracheally and then challenged intravenously. These responses consisted predominantly of indirect pfc.

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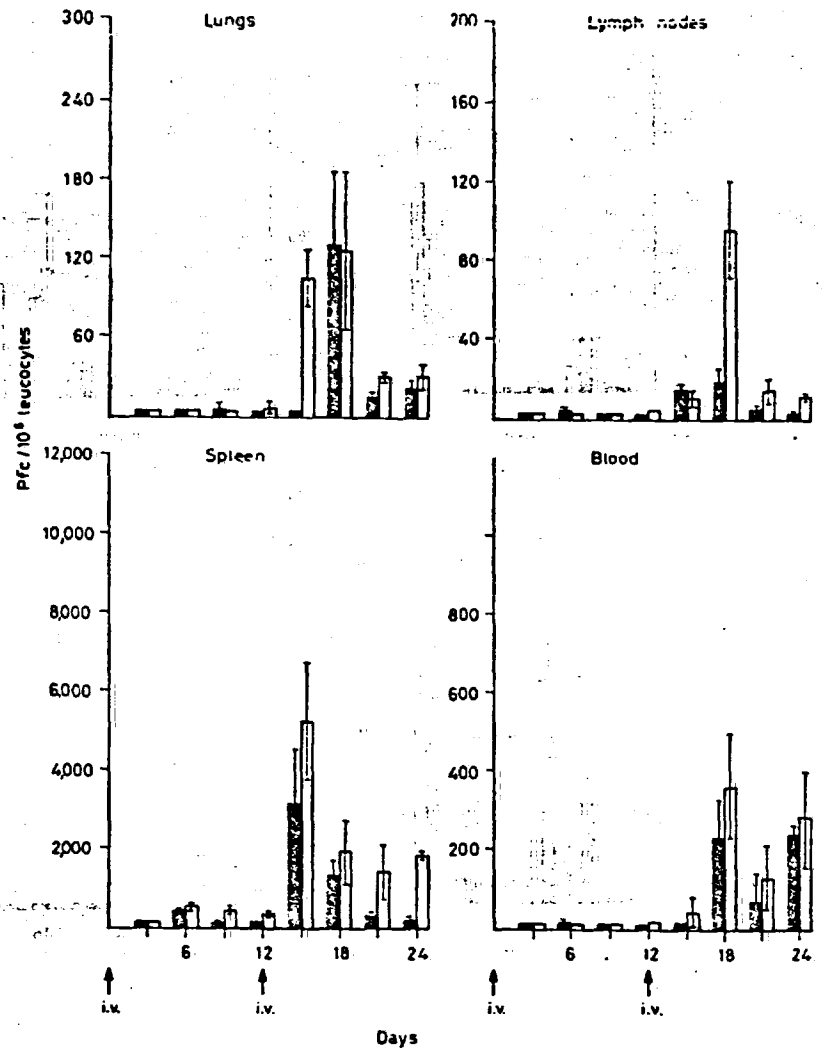

FIGURE 5 Plaque-forming-cell response in mice immunized with two intravenous (IV) inoculations of 10^8 SRBC; direct pfc ■ indirect pfc □ , ↑ indicates time of inoculation.

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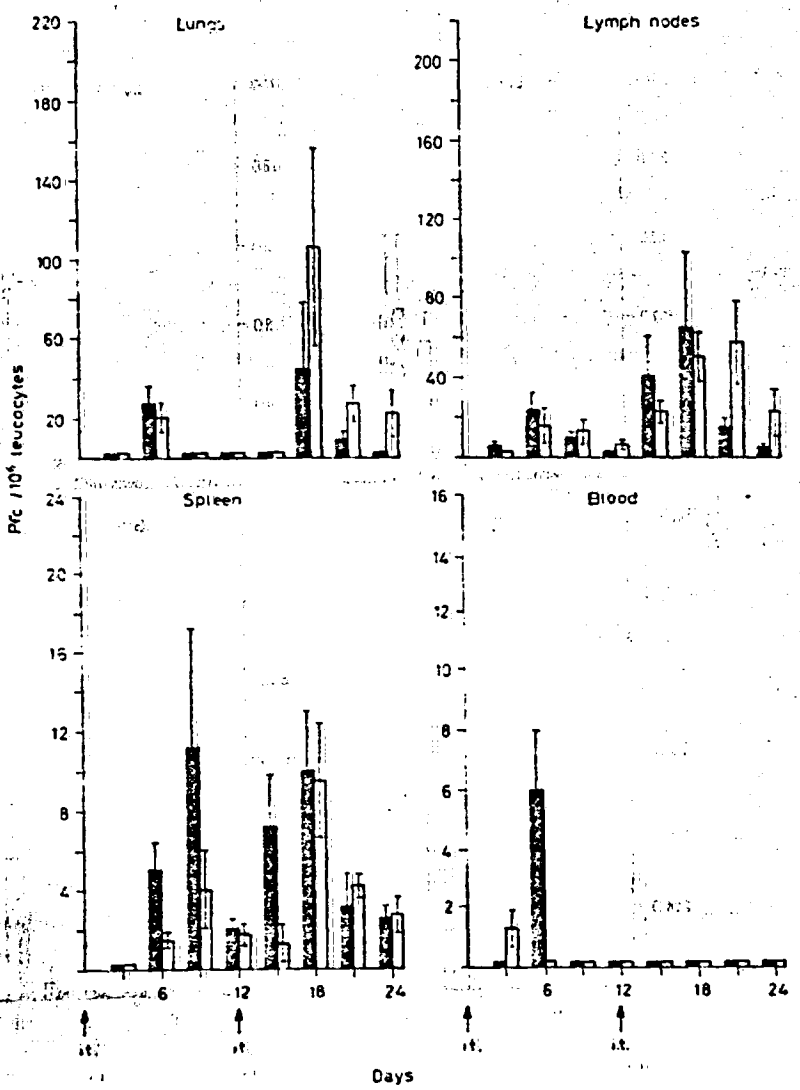

FIGURE 6 Plaque-forming-cell response in mice immunized with two intratracheal (IT) inoculations of 10^8 SRBC; direct pfc ■ indirect pfc □ , † indicates time of inoculation.

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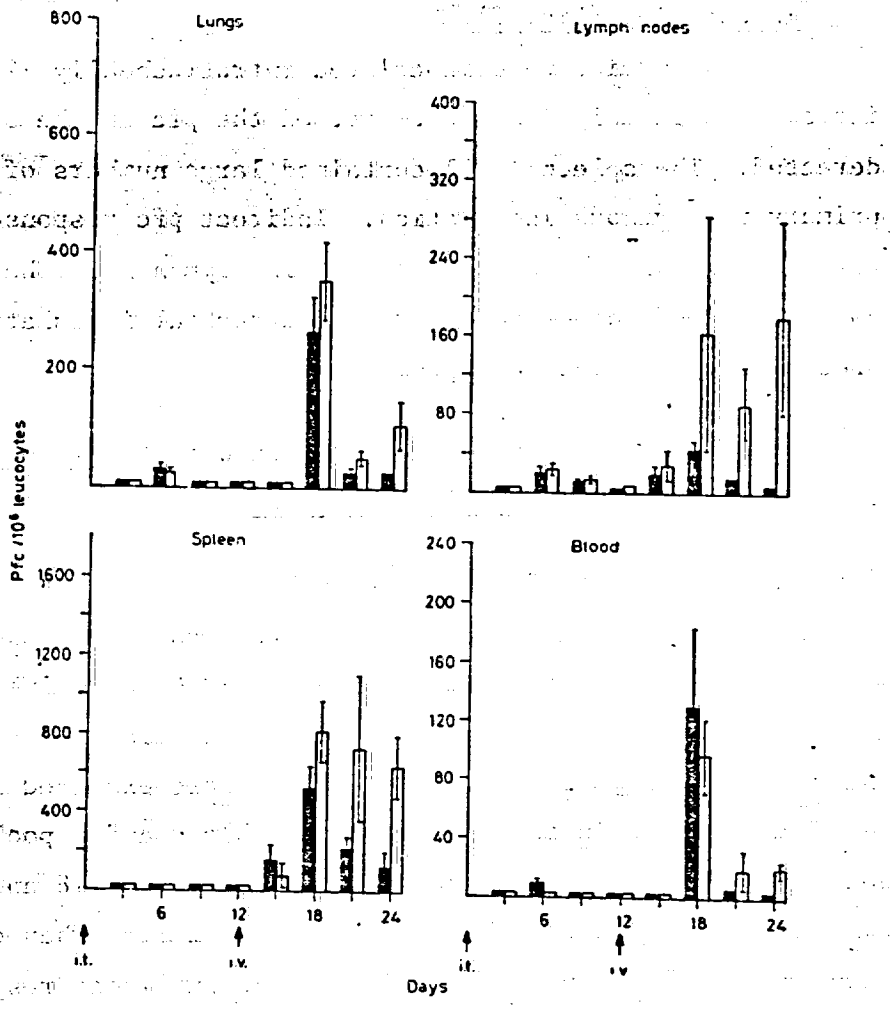

FIGURE 7 Plaque-forming-cell response in mice immunized with an intratracheal (IT) then an intravenous (IV) inoculation of 10^8 SRBC; direct pfc ■ , indirect pfc □ , † indicates time of inoculation.

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Although pfc persisted in the circulation after these inoculations, this response was not significantly correlated ($r = 0.46$) with the lung reaction. The lungs also contained more pfc/10^6 leucocytes than the blood. Sufficient priming of the spleen occurred after intratracheal challenge to produce an intense reaction after intravenous challenge.

(iv) Intratracheal inoculation of mice sensitized by intravenous inoculation (Fig. 8)

When mice were inoculated intratracheally 12 days after an intravenous inoculation, no effect on the pfc in the spleen could be detected. The spleen still contained large numbers of pfc from the primary intravenous inoculation. Indirect pfc responses were detected in the blood and the lungs. The lymph nodes had a response no greater than after a primary intratracheal inoculation, consisting predominantly of indirect pfc.

C. Humoral immune responses of mice exposed to cigarette smoke to intratracheal inoculations of SRBC

(a) Primary and secondary pfc response

C57Black mice were exposed to cigarette smoke for 26 weeks and inoculated intratracheally with 10^8 SRBC in 0.01ml of PBS and the smoking regime continued. A second inoculation was given, where necessary, on day 12. Groups of 5 mice were examined for direct and indirect pfc in the lungs, spleen, blood and a pool of the mediastinal and cervical lymph nodes at 7, 9, 12, 15, 18 and 21 days after the first inoculation. The results were similar when considered per organ or per 10^6 leucocytes. The only significant response found in the blood was in age-matched control mice 7 days after the inoculation (5 ± 2 direct pfc/10^6 leucocytes). All the organs of mice exposed to cigarette smoke had modified pfc responses. The primary response in the lungs (Fig. 9) was virtually abrogated and although it was initiated in the spleen (Fig. 9) and lymph nodes (Fig. 9) it was reduced and short lived. After a second inoculation the response in the lungs of cigarette-smoke exposed mice was delayed and although a large number of pfc eventually developed there was a large participation of direct pfc. The secondary response in the lymph nodes and spleen was marked by a depression in the indirect pfc response.

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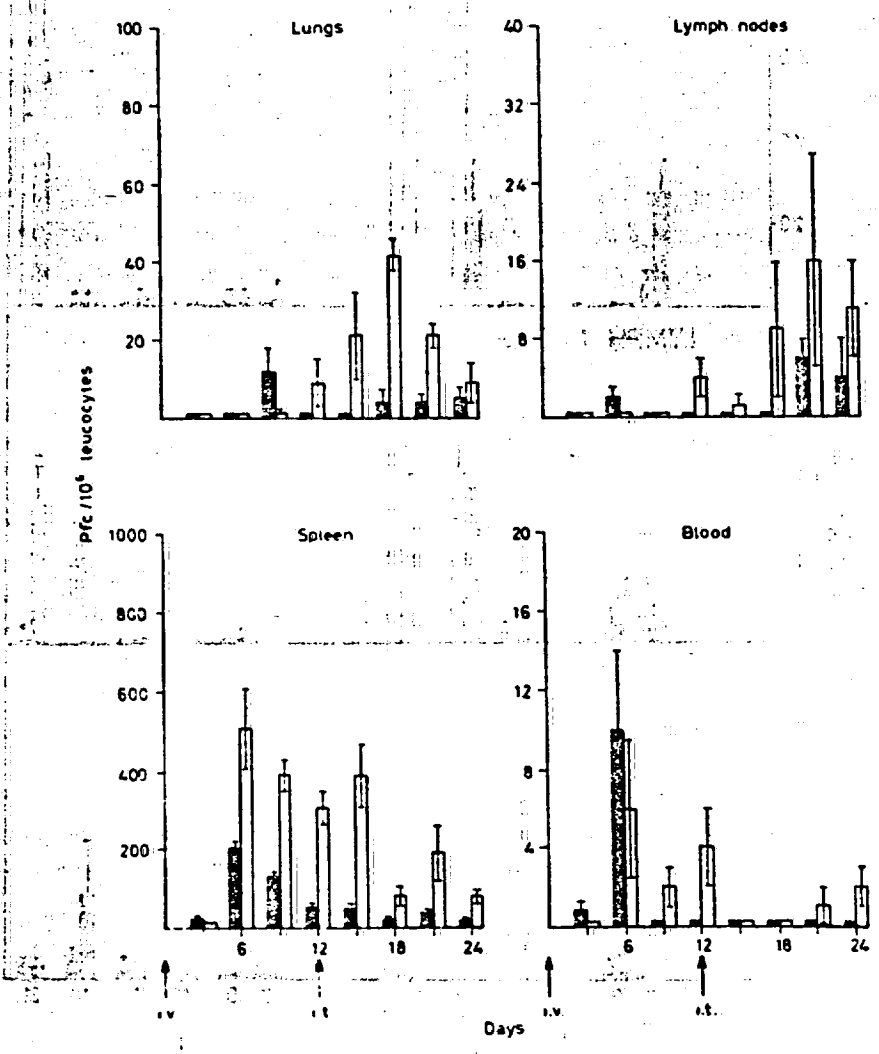

FIGURE 8 Plaque-forming-cell response in mice immunized with an intravenous (IV) then an intratracheal (IT) inoculation of 10^8 SRBC; direct pfc ■ , indirect pfc □ , ↑ indicates time of inoculation.

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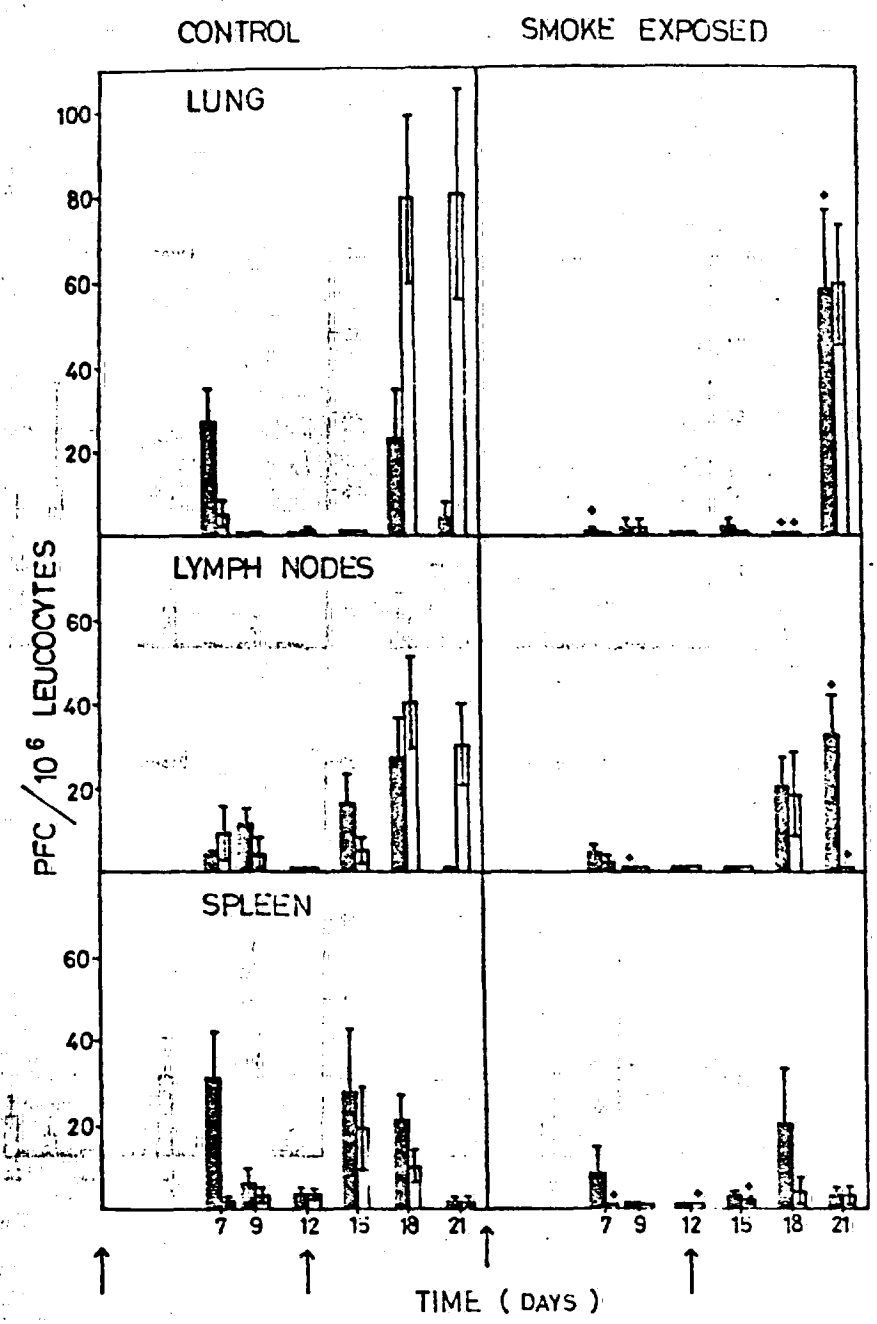

FIGURE 9 Primary and secondary plaque-forming-cell response in the spleen, lymph nodes and lungs of mice exposed to cigarette smoke for 26 weeks and control mice after intratracheal inoculations of SRBC. ↑ time of inoculation, direct pfc ■, indirect pfc □. Each result is the mean ± S.E. from groups of 5 mice. The significance of the differences between the cigarette smoke exposed and control mice was judged by the Student's t test: • $p<0.05$.

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(b) The development of the alterations in the primary immune response

The time course of the changes occurring in the primary immune response of the spleen, lungs and the pool of mediastinal and cervical lymph nodes was examined in C57Black mice exposed to the cigarette smoke for periods of up to 42 weeks. The direct and indirect pfc responses were determined in groups of mice 7 days after an intratracheal inoculation of 10^8 SRBC. The smoking regimes were continued throughout the immunization period. The primary responses of groups of five mice exposed to cigarette smoke for 0, 9, 36, 120, 182 and 294 days were determined. The responses of the mice exposed to cigarette smoke, expressed as a percentage of the responses of age-matched-control mice, are shown in Figs. 10, 11 and 12. No significant change was found in the pfc responses of mice only exposed to the cigarette smoke after the inoculation of SRBC.

The influence of the exposure to cigarette smoke on the primary pfc response in the lung (Fig. 10) was apparent after 9 days. At this time the number of direct and indirect pfc detectable in the lungs fell to 30% of control values. After 182 days of smoke exposure the response in the lungs was less than 5% of the responses of control mice.

In the lymph nodes (Fig. 11) direct pfc response showed a rapid rise reaching a plateau between 96 and 120 days. This response then rapidly declined to show a severe depression by 182 days. The indirect pfc response in the lymph nodes was not stimulated to the same degree as the direct pfc response, but was depressed by prolonged exposure to cigarette smoke.

The direct pfc responses in the spleen became irregular after exposure to cigarette smoke, with many animals having high responses, and there was no consistent deviation from control levels until after 294 days smoking when a marked depression was apparent. The indirect pfc responses were similar, however the number of indirect pfc were low.

(c) Serum antibody

C57Black mice were exposed to cigarette smoke for 19 weeks and then inoculated intratracheally with 10^8 SRBC. The serum

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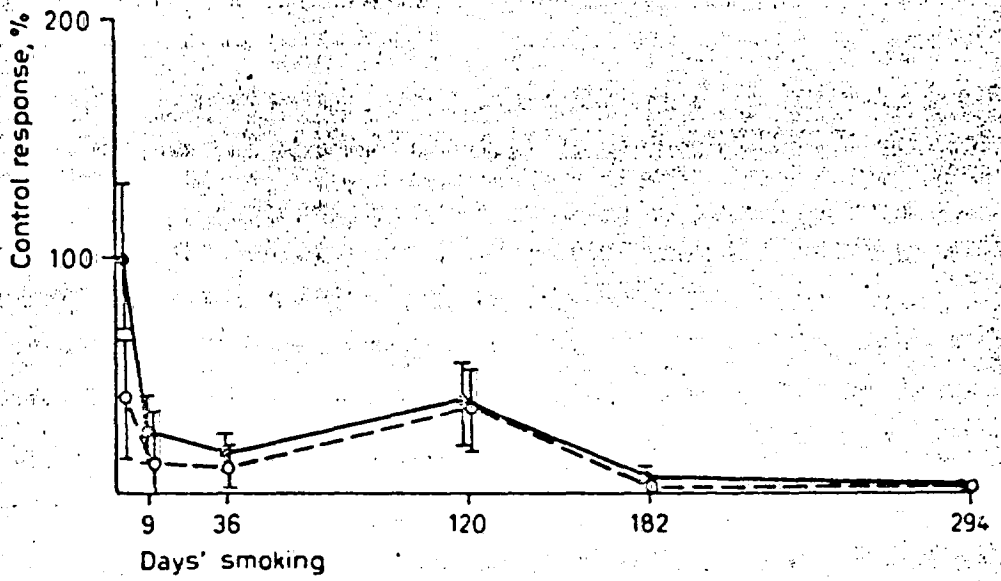

FIGURE 10 Effect of cigarette-smoke exposure on the plaque-forming-cell response in the lung to an intratracheal inoculation of SRBC. Mice were exposed to cigarette smoke for 0, 9, 36, 120, 182 and 294 days before immunizing intratracheally with 10^8 SRBC. "0" indicates exposure for the 7 days following immunization. The mean (± S.E.) number of pfc/10^6 leucocytes appearing 7 days after the inoculation is shown as a percentage of the response of age-matched controls for direct ● and indirect o, pfc. Control mice had an average of 22 direct and 13 indirect pfc/10^6 leucocytes. As judged by Student's t test the smoke exposed and control animals had a significantly different direct and indirect pfc response ($p<0.05$) on days 9, 36, 120, 182 and 294.

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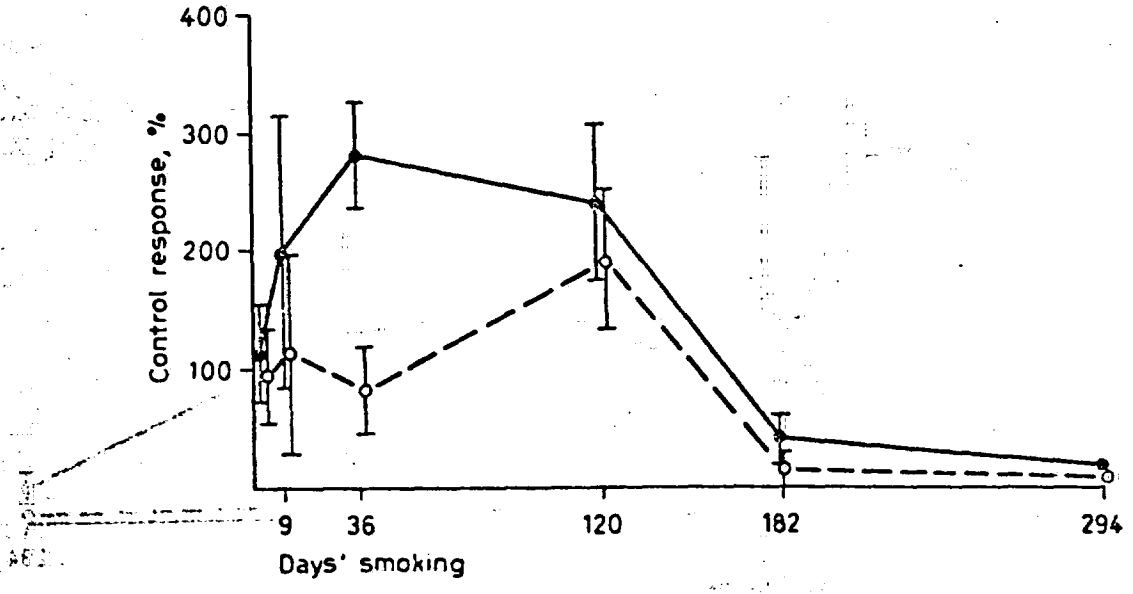

FIGURE 11 Effect of cigarette-smoke exposure on the plaque-forming cell response in the cervical and mediastinal lymph nodes to an intratracheal inoculation of SRBC. Mice were exposed to cigarette smoke for 0, 9, 36, 120, 182 and 294 days before immunizing intratracheally with 10^8 SRBC. "0" indicates exposure for the 7 days following immunization. The mean (± S.E.) number of pfc/10^6 leucocytes appearing 7 days after the inoculation is shown as a percentage of the response of age-matched controls for direct ●, and indirect o, pfc. Control mice had an average of 33 direct and 46 indirect pfc/10^6 leucocytes. As judged by Student's t test the smoke exposed and control animals had a significantly different ($p<0.05$) direct pfc response on days 9, 182 and 294 and indirect response on day 294.

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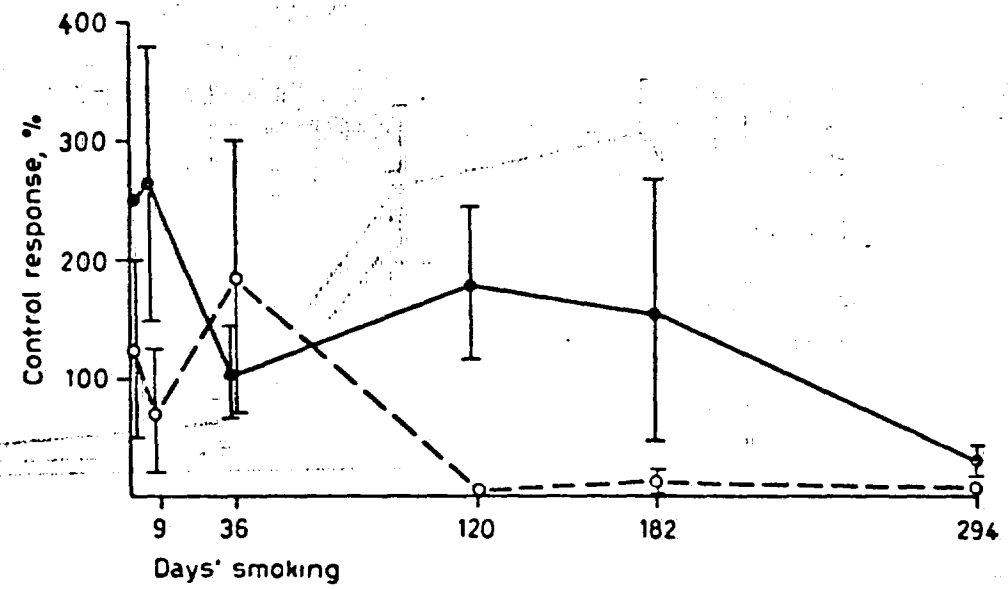

FIGURE 12 Effect of cigarette-smoke exposure on the plaque-forming cell response in the spleen to an intratracheal inoculation of SRBC. Mice were exposed to cigarette smoke for 0, 9, 36, 120, 182 and 294 days before immunizing intratracheally with 10^8 SRBC. "0" indicates exposure for the 7 days following immunization. The mean (± S.E.) number of pfc/10^6 leucocytes appearing 7 days after the inoculation is shown as a percentage of the response of age-matched controls for direct •, indirect o, pfc. Control mice had an average of 13 direct and 2 indirect pfc/10^6 leucocytes. As judged by Student's t test the smoke exposed and control animals had a significantly different ($p<0.05$) direct pfc response on day 294.

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haemagglutinating and haemolytic antibody titres were measured 7 days after inoculation. The mice exposed to cigarette smoke had lower haemagglutinin and haemolysin titres (Table 11).

(d) Recovery of the pfc response after the cessation of cigarette smoking

C57Black mice were exposed to cigarette smoke for 42 weeks and then left for a further 16 weeks without cigarette smoke exposure. Their primary pfc response in the spleen, lung, and a pool of mediastinal and cervical lymph nodes was then measured 7 days after an intratracheal inoculation of 10^8 SRBC. Their response, expressed as a percentage of the response of age-matched-control mice, is shown in Table 12 compared to the response of mice exposed to cigarette smoke for 42 weeks. Recovery was evident in the direct pfc response in the lungs and lymph nodes. Mice discontinuing cigarette smoking still had low responses in the spleen. The indirect pfc response showed a similar trend but the numbers of indirect pfc in a primary response were low.

D. Humoral immune response of mice exposed to cigarette smoke to intraperitoneal inoculations of SRBC

Groups of C57Black mice were exposed to cigarette smoke for 1, 17 or 38 weeks, and their primary and secondary pfc and antibody responses to intraperitoneal inoculations of SRBC determined. The exposure to cigarette smoke was continued throughout the experiment. To examine the pfc and serum antibody responses to SRBC the mice were inoculated with 10^8 SRBC and where required a secondary response was elicited with another inoculation of 10^8 SREC on day 12. Groups of 5 mice were examined on day 4, 8, 16 and 20 after the first inoculation. The animals were exsanguinated and the serum stored at $-20^{\circ}C$ before assaying for antibody activity.

The spleen and a pool of cervical and mediastinal lymph nodes were examined for direct and indirect pfc. The pfc responses when considered per organ or per 10^6 leucocytes were similar. The direct and indirect pfc per 10^6 leucocytes in the spleens and lymph nodes are shown in Figs. 13 and 14.

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TABLE 11 Serum antibody titres of mice exposed to cigarette smoke for 19 weeks and inoculated intratracheally with SRBC

	Smoke exposed	Control
Haemolysin	0.22 ± 0.14	1.25 ± 0.45
Haemagglutinin	1.45 ± 0.24	3.4 ± 0.67

Each result is the mean ± S.E. of the $\log_2$ titre from 9 or 10 mice. The haemolysin and haemagglutinin titres of the mice exposed to cigarette smoke were significantly different from the titres of control mice as judged by Student's t test, $p<0.05$.

TABLE 12 Primary pfc responses of mice to an intratracheal inoculation of SRBC after discontinuing cigarette smoke exposure

	42 weeks smoke exposure		42 weeks smoke exposure and 16 weeks "recovery"	
	Direct pfc	Indirect pfc	Direct pfc	Indirect pfc
Lung	1.2 ± 0.3	0	** 75 ± 14.9	90 ± 58
Lymph nodes	11 ± 8.1	3 ± 3	* 69 ± 22.5	33.5± 15.2
Spleen	23 ± 8.0	0	(1) 41 ± 31.0	(2) 273 ± 256

The pfc responses expressed as a percentage of age matched controls. Except where indicated each result is the mean ± S.E. of the values from 5 mice: (1) 9 mice, (2) 6 mice. The significance of the differences between the smoke exposed group and the group discontinuing smoking was examined by Student's t test, * $p<0.05$, ** $p<0.001$

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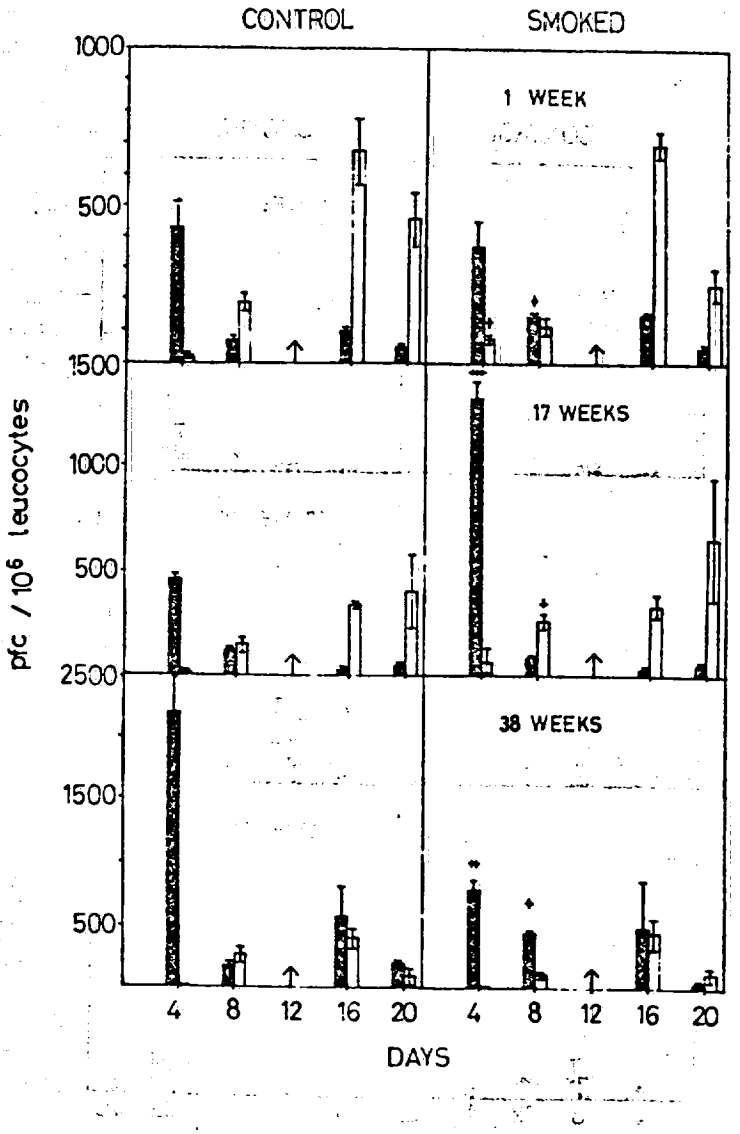

FIGURE 13 Primary and secondary pfc response in the spleen of mice exposed to cigarette smoke for 1, 17 and 38 weeks and immunized with two intraperitoneal inoculations of 10^8 SRBC. Each result represents the mean ± S.E. of the values from 5 mice; direct pfc ■, indirect pfc □, ↑ inoculation. The significance of the difference between the cigarette smoke exposed and control animals was determined by Student's t test; + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$.

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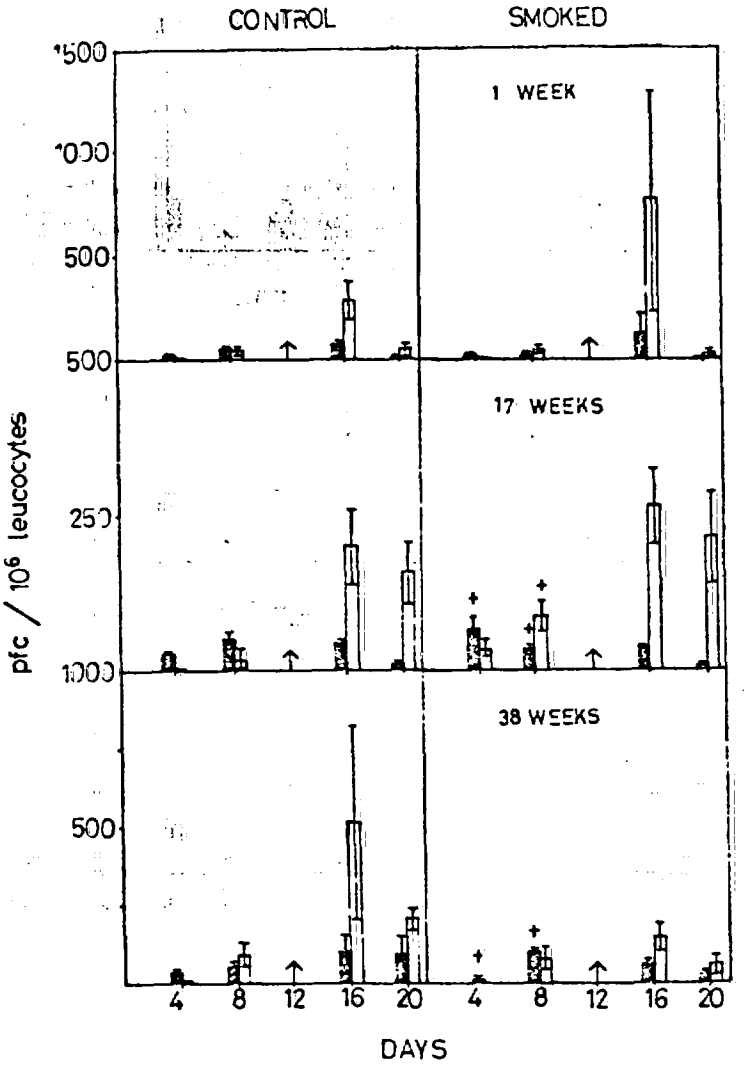

FIGURE 14 Primary and secondary pfc response in the mediastinal and cervical lymph nodes of mice exposed to cigarette smoke for 1, 17 and 38 weeks and immunized with two intraperitoneal inoculations of 10^8 SRBC. Each result represents the mean ± S.E. of the values from 5 mice; direct pfc ■, indirect pfc □, † inoculation. The significance of the difference between the cigarette smoke exposed and control animals was determined by Student's t test; + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$.

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After 1 week of smoke exposure the only differences between the smoke-exposed and control animals were that the animals exposed to cigarette smoke had a higher number of direct pfc in the spleen on day 8 and a higher number of indirect pfc in the spleen on day 4. Following 17 weeks of smoke exposure the mice had a highly elevated primary pfc response in the spleen and the lymph nodes. There was little difference between the secondary responses of the smoke-exposed and control animals.

The pfc response of the mice exposed to cigarette smoke for 38 weeks and the corresponding control mice had a high number of direct pfc in the spleen after the primary and secondary inoculation. However the mice exposed to cigarette smoke clearly had a depressed direct and indirect pfc response in the spleen after the primary inoculation. In the lymph nodes the primary pfc response of the mice exposed to cigarette smoke was delayed compared to the controls; the control mice had a significantly higher pfc response on day 4 but a significantly lower pfc response on day 8. No differences in the secondary responses were evident. The serum haemolytic and haemagglutinating antibody titres for the primary and secondary responses (Fig. 15) showed a stimulation of the primary haemolytic antibody response after 17 weeks exposure to cigarette smoke. A depression of the haemolytic antibody response after the primary and secondary inoculations was evident in mice exposed to cigarette smoke for 38 weeks.

E. Natural antibody producing cells to SRBC in mice exposed to cigarette smoke

A group of C57Black mice was exposed to cigarette smoke for 16 weeks and then the cervical and mediastinal lymph nodes and the spleen examined for 'natural' pfc to SRBC. Corresponding unimmunized control animals were also examined. No significant difference in the numbers of pfc in control mice and mice exposed to cigarette smoke was evident (Table 13).

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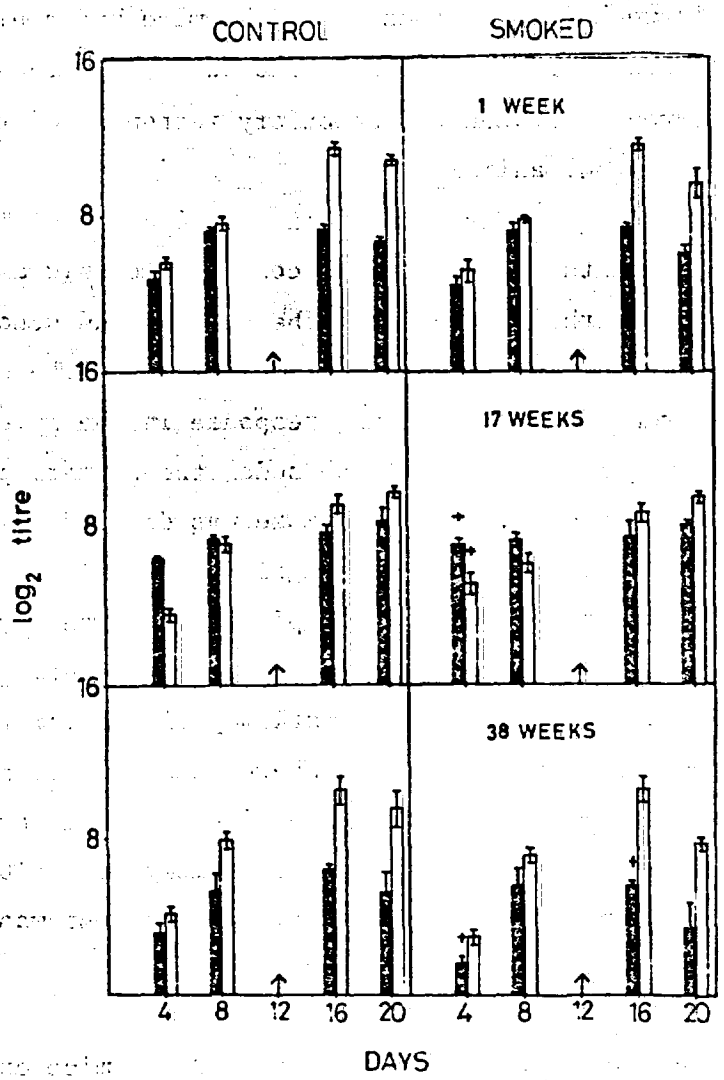

FIGURE 15 Primary and secondary serum antibody response of mice exposed to cigarette smoke for 1, 17 and 38 weeks and immunized with intraperitoneal inoculations of 10^8 SRBC. Each result represents the mean ± S.E. of the values from 5 mice; haemolysin ■, haemagglutinin □, ↑ inoculation. The significance of the differences between the smoke exposed and control animals was determined by Student's t test; + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$.

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TABLE 13 Plaque forming cells to SRBC in unimmunized mice exposed to cigarette smoke for 16 weeks

	pfc/10^6 leucocytes			
	Smoke exposed		Control	
	Direct pfc	Indirect pfc	Direct pfc	Indirect pfc
Spleen	0.2 ± 0.2	0	0.8 ± 0.4	0.2 ± 0.29
Lymph nodes	0	0	0.2 ± 0.2	0.2 ± 0.2

Each result is the mean ± S.E. of the values from 5 mice. As judged by the Student's t test there was no significant difference between the cigarette-smoke exposed and control mice. Mice were exposed to cigarette smoke for 16 weeks and then the 'natural' number of pfc in the spleens and pool of mediastinal and cervical lymph nodes was determined.

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F. The immune response of mice exposed to cigarette smoke to polyvinylpyrrolidone (PVP)

Groups of C57Black mice were exposed to cigarette smoke for 8, 15, 30 and 41 weeks. The mice were then inoculated intravenously with 0.2µg of PVP (MW 360,000) in 0.1ml of PBS. One week after this inoculation the serum-antibody titres were measured by the indirect haemolysis of PVP coated SRBC. The exposure to cigarette smoke was continued throughout all inoculation regimes. The indirect-haemolytic-antibody titres (Table 14) did not show any significant difference between mice exposed to cigarette smoke and control animals. The serum of normal mice and unimmunized mice exposed to cigarette smoke for 15 weeks did not have a detectable titre to PVP.

G. The effect of cigarette-smoke solutions on antibody-producing cells and antibody activity *in vitro*

(a) Antibody-forming cells

Plaque-forming cell preparations were prepared from the spleens of mice, 4 - 6 days after an intraperitoneal inoculation of 10^8 SRBC. The cell preparations were incubated in final dilutions of 1/2.5, 1/10 and 1/100 of cigarette-smoke solution and incubated for 0, 1, 2 and 4 hours at 37°C before assaying for pfc. Except for the assay performed immediately after the addition of smoke solution, the cells were centrifuged (200g, 5 minutes) and resuspended in fresh medium before the pfc assay. The cigarette-smoke solution had no immediate effect on the number of pfc (Fig. 16), but after continued incubation the number of pfc showed a transient depression. The extent of the inhibition and rate of recovery was dependent on the concentration of smoke solution. A further decrease rather than a recovery of pfc numbers was found if cells that had been incubated in a 1/10 smoke solution for 1 hour were retreated with a fresh 1/10 smoke solution for another hour.

(b) Antibody activity

The effect of cigarette-smoke solutions on the antibody activity of 7S and 19S fractions of rabbit anti-SRBC serum prepared

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TABLE 14 Serum antibody response of mice chronically exposed to cigarette smoke to polyvinylpyrrolidone

Weeks exposure[1]	Log_2 Antibody titre Smoke exposed	Control
8	2.6 ± 0.40	1.4 ± 0.51
15	2.8 ± 0.20	2.4 ± 0.24
30	[2] 3.0 ± 0.45	[2] 3.3 ± 0.21
41	[3] 2.6 ± 0.34	[3] 2.0 ± 0.71

Each result is the mean ± S.E. of log_2 antibody-titre in the serum one week after the inoculation of PVP. Each group had 5 mice except where indicated: (2) 6 mice, (3) 3 mice. (1) Weeks exposure to cigarette smoke before the inoculation of PVP.
No significant difference between cigarette smoke exposed and control animals was found as judged by Student's t test.

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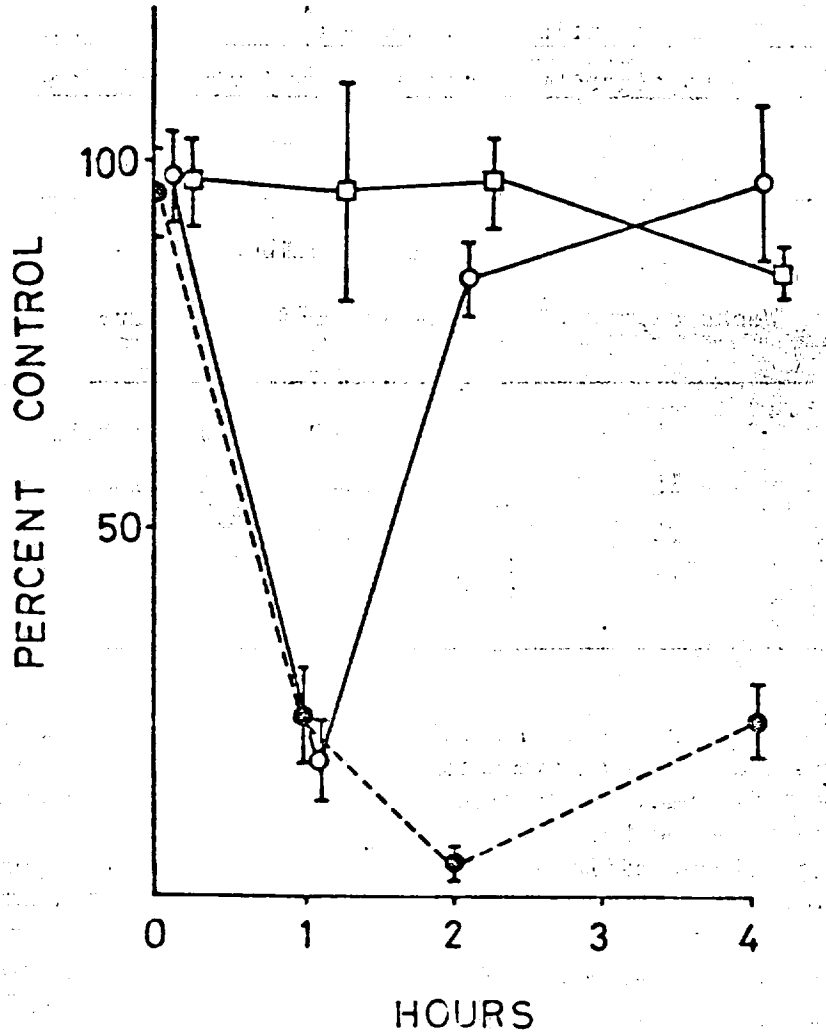

FIGURE 16 Effect of cigarette-smoke solutions on plaque-forming cells *in vitro*. Each result is the mean ± S.E. of 6 cultures. The results show the number of pfc found in cultures of antibody-forming cells, when assayed at 0, 1, 2 and 4 hours after the addition of smoke solution to the cultures, as a percentage of control values. Three dilutions of smoke solution were used; ● 1/2.5, o 1/10, □ 1/100.

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by Sephadex gel filtration, was examined. Haemagglutinating and haemolytic antibody activity was studied in both fractions. The fractions were incubated at 37°C in a final concentration of 1/2.5 of a fresh stock smoke solution for 1 hour. More fresh smoke solution was then added to a final concentration of 1/2.5 of the fresh smoke solution and the fractions incubated for another hour. The haemagglutinating and haemolytic antibody titres were measured after the second hour and compared to controls (Table 15). The smoke solutions did not affect the titres. The addition of smoke solution to titrations using reagents not preincubated with smoke solutions did not affect the titres.

H. Discussion

(a) Humoral response of normal mice to local and systemic inoculation of SRBC

After an intratracheal inoculation of SRBC, pfc were found in the lungs and regional lymph nodes, the spleen and in the peripheral blood. However the axillary lymph nodes, did not respond significantly to the intratracheal inoculation. Pfc could be found in the lungs and lymph nodes after either topical or systemic immunization but generally the response in the respiratory system was greatest after local immunization.

It is important to consider whether or not the pfc detected in the lungs were part of the population circulating in the blood. This was clearly not the case where the immunization regime produced little or no response in the blood, but the interpretation was not so obvious when large responses occurred in both organs. In many of the cases where large responses were found in the blood, the concentration of pfc (per 10^6 leucocytes) in the lung was less than 10% of the concentration in the blood. If this percentage was taken as the contribution from freely circulating cells, the remaining pfc found in the lung must have had some closer association with the respiratory system.

Following two intravenous inoculations of SRBC, large numbers of pfc were found in the blood and the lung. The responses in these organs were highly correlated. In the lung, the number of pfc/10^6 leucocytes reached 50% of that found in the blood. As

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TABLE 15 Effect of cigarette-smoke solutions on antibody activity

	Smoke exposed		Control	
Fraction	Haemagglutinin	Haemolysin	Haemagglutinin	Haemolysin
19S	2.3 ± 0.3	6 ± 0	2.3 ± 0.3	6 ± 0
7S	2.0 ± 0	2.0 ± 0	2.0 ± 0	2 ± 0

Each result is the mean ± S.E. of the result of 3 titrations of the antibody activity in fractions exposed to cigarette smoke solutions for 2 hours.

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previously discussed, only a small proportion of these could be accounted for in terms of transient circulating cells. It therefore appeared that the majority of pfc in the lungs were seeded from the circulation, or had been initiated locally. Hiramoto, Habeeb and Hamlin (1970), and Hiramoto and Hamlin (1970) have reported that the lung could be an extralymphoid site of antibody production after systemic immunization. The antibody-forming cells were found to accumulate around the pulmonary vessels and alveolar walls (Marshall and White 1950; Hiramoto, Habeeb and Hamlin 1970). In contrast to the lung, the mediastinal and cervical lymph nodes responded to systemic immunization but this response did not appear to be associated with the pfc found in the blood.

Intratracheal inoculation of SRBC produced a response in mice which resulted in pfc being detected in the blood, spleen, lung and the mediastinal and cervical lymph nodes. After this inoculation about 200 pfc/lymph node were produced. Only a low number were found in the blood. It was difficult to estimate the total number of cells in the lung but in the order of 100 pfc/lung were probably produced. The lung, the lymph nodes and the spleen showed a secondary response to a second intratracheal inoculation. The quantitative and temporal aspects of the response in the lung indicated that it was locally produced. An intratracheal inoculation of SRBC has been found to induce a local antibody response in canine tracheal-pouches (Lieberman *et al* 1972). After local immunization with SRBC, pfc have also been found in the endobronchial lavages of rabbits (Holub and Hauser 1969; Ford and Kuhn 1973a,b).

When the local response induced by intratracheal immunization was boosted by a systemic inoculation, large responses were detected in the lungs and the lymph nodes as well as a large number of pfc in the blood. The response in the lung had a higher concentration of pfc than the blood and the responses were not significantly correlated. The intravenous inoculation apparently boosted the response centralised in the lungs and lymph nodes. However seeding from the spleen cannot be eliminated. Mice preimmunized systemically responded to the local inoculation by primarily producing an indirect pfc response, little greater in magnitude to the primary response to a local inoculation.

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The intratracheal inoculation of SRBC produced a pfc response which appeared to be localised in the lung and in the lymph nodes and spleen, and provided a method for assessing the immune response in the respiratory tract.

(b) The effect of exposure to cigarette smoke on the humoral immune response

The humoral immune responses of mice exposed to cigarette smoke to intratracheal and intraperitoneal inoculations were measured. The exposure to cigarette smoke produced almost identical alterations to the response to locally or systemically administered SRBC. Cigarette-smoke exposures for up to 17 weeks stimulated the primary pfc responses in the spleen and lymph nodes to both inoculation regimes. The statistical significance of the stimulation of the primary pfc response in the spleen after intratracheal inoculation has been established (Nulsen, Holt and Keast 1974). After continued exposures to cigarette smoke the primary immune response to locally and systemically inoculated SRBC was depressed. These included the primary pfc responses in the lung, lymph nodes and spleen as well as serum antibody; both haemagglutinating and haemolytic antibody titres elicited after either local or systemic inoculation were decreased. The depression of the pfc response in the lungs occurred soon after the commencement of smoke exposure and a phase of enhanced response was not detected. However mice inoculated intratracheally with SRBC and then exposed to cigarette smoke did not have depressed pfc responses. The only alteration in the pfc and antibody responses of mice to systemic inoculation of SRBC, produced by one week of exposure to cigarette smoke, was a slight increase in the pfc response of the spleen. Mice chronically exposed to cigarette smoke could at least partially recover their ability to respond to SRBC when the exposure to cigarette smoke was discontinued.

As the primary responses of mice exposed to cigarette smoke and control mice were different, the effect of a second inoculation of immunogen can not be strictly compared. Even so, it appeared that mice exposed to cigarette smoke were able to respond normally to continued antigenic challenge. The secondary pfc responses to intraperitoneal inoculations of SRBC were almost identical in control mice and mice exposed to cigarette smoke. This occurred regardless of whether the primary response of the mice exposed to

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cigarette smoke was stimulated or depressed. The secondary pfc responses of mice exposed to cigarette smoke, to intratracheal inoculations of SRBC, were still lower than the responses of control mice and had an abnormally large participation of direct pfc. However the responses after a second intratracheal inoculation were considerable higher than the primary responses and it appears probable that the difference in the effect of exposure to cigarette smoke on the secondary intratracheal and intraperitoneal responses was only due to the ability of the intraperitoneal inoculations to elicit a more vigorous immune response. It should be noted that a small decrease in the serum antibody response to a second intraperitoneal inoculation was found in mice chronically exposed to cigarette smoke.

As SRBC have been found to be T-cell dependent immunogens, (Davies *et al* 1971; Playfair 1971) serum antibody responses to an immunogen not requiring T-cells, PVP (Andersson and Blomgren 1971), was also measured. No significant difference could be found between the response of control mice and mice exposed to cigarette smoke for periods up to 41 weeks. This does not necessarily imply that either T-cell function is impaired or B-cell function is not impaired in mice exposed to cigarette smoke. The mice exposed to cigarette smoke could have a defect in macrophage function; there is evidence showing that macrophages are required for lymphoid cell co-operation (Feldman 1972; Waldron, Horn and Rosenthal 1973). It is also possible that a defect in T-cell and B-cell co-operation could be caused by a defect of B-cells. However the response to PVP and the secondary responses to SRBC indicate that it was possible to stimulate a normal humoral immune response in cigarette-smoke-exposed mice.

Experiments with solutions of cigarette smoke showed that haemagglutinating and haemolytic activities of 19S and 7S antibodies were resistant to the acute effects of cigarette smoke. It would, however, be reasonable to expect that cigarette smoke could have a direct inhibitory action on the function of antibody-forming cells in the lung. Since human cigarette smokers typically smoke several cigarettes daily, their respiratory tract would be subject to the direct effects of cigarette smoke for a considerable period of time. The exposure to cigarette smoke did not affect the antibody-producing cell response in the lungs of mice exposed to cigarette smoke after

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immunization. However, the mice in these experiments were not exposed to cigarette smoke on the day of assay.

SECTION 2 CELL-MEDIATED IMMUNITY

A. Introduction

Cell-mediated immune reactions result from the action of antigen on T-cells which then respond by blast transformation, proliferation and the production of substances which participate in the immune reaction (Bellanti 1971; World Health Organization 1973). These substances may be effector molecules acting on tumour cells and infectious agents, or mediator molecules, which recruit other cells into the immune reaction. The major collaborating cells in cell-mediated immune reactions appear to be the mononuclear phagocytes. A large number of biological activities of products from activated lymphocytes have been reported and at least several different molecules are responsible for the activities (David 1971; World Health Organization 1973). The incorporation of H^3-thymidine into lymphocytes in the presence of PHA is a measure of the proliferation of T-cells and has been recognized as an *in vitro* correlate of the potential to mount cell-mediated immune reactions (Rodey and Good 1969; Adler *et al* 1970; Bellanti 1971; Keast and Bartholomaeus 1972; Stobo, Rosenthal and Paul 1972; World Health Organization 1973). The potential of mice exposed to cigarette smoke to elicit cell-mediated immune reactions was estimated by measuring the incorporation of H^3-thymidine into lymphocytes incubated with PHA.

B. Response of lymphocytes from mice exposed to cigarette smoke to PHA

Groups of C57Black mice were exposed to cigarette smoke for 5, 15, 25 and 35 weeks. The responsiveness of the lymphocytes from the spleen, peripheral blood and a pool of the cervical and mediastinal lymph nodes of individual mice to PHA was determined by a micromethod measuring the incorporation of H^3-thymidine into the DNA of lymphocytes incubated with various concentrations of PHA (Keast and Bartholomaeus 1972). Tests on the lymphocytes from 6 - 12

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week old and 40 week old controls were also performed. Triplicate cultures at each PHA concentration were set up and the means and S.E. for groups of three animals at each exposure time were determined.

In the experiments, the responsiveness of the lymphocytes recovered from the blood and spleen to PHA exhibited similar changes throughout the period of cigarette smoke exposure (Figs. 17, 18). After five weeks exposure to cigarette smoke the responsiveness of the lymphocytes from both these organs was depressed. At weeks 15 and 25 a transient recovery of responsiveness was noted. By the 35th week of exposure the peak response of the lymphocytes from each organ was depressed. However, only the peak response of lymphocytes from blood was significantly depressed below the peak response of the lymphocytes from 40 week old controls (Fig. 17). There was a tendency for the splenic lymphocytes to respond maximally to higher levels of PHA than the 40 week old controls.

The lymph node cells displayed an enhanced response to PHA after 5 weeks cigarette smoke exposure but after 15 weeks the lymphocytes would only respond to high concentrations of PHA. A large response was obtained with the lymph node lymphocytes from mice exposed for 25 weeks but after 35 weeks exposure to cigarette smoke responsiveness to PHA was markedly depressed. The peak response of the lymph node lymphocytes from the mice exposed for 35 weeks was significantly lower than the response of the lymph node lymphocytes from the 40 week control mice. Hence the ultimate result of the prolonged cigarette smoke exposure was a depression of the responsiveness of lymphocytes to PHA, particularly in the lymph nodes draining the respiratory system (Fig. 19).

C. Discussion

The incorporation of H^3-6-thymidine into lymphocytes in the presence of PHA is a measure of the proliferation of T-cells (Rodey and Good 1969; Adler *et al* 1970; Bellanti 1972; Keast and Bartholomaeus 1972; Stobo, Rosenthal and Paul 1972; World Health Organization 1973). This is a function of the number of T-cells as well as their ability to divide after contact with the mitogen. T-cells form the major proportion of the recirculating lymphocytes and procedures which deplete recirculating lymphocytes diminish cell-mediated immune

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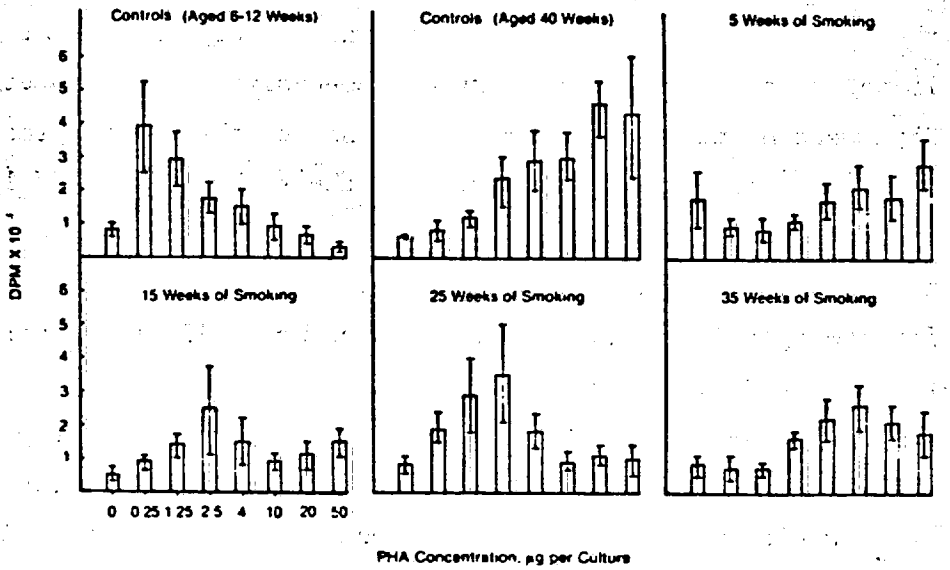

FIGURE 17 The PHA response of peripheral blood lymphocytes. Each point represents the mean ± S.E. derived from the cultures from three mice. The responses of 6 - 12 week old and 40 week old control mice as well as animals exposed to cigarette smoke for 5, 15, 25 and 35 weeks are shown. The peak response after 35 weeks smoking was significantly different from that of control mice as determined by Student's t test ($p<0.05$).

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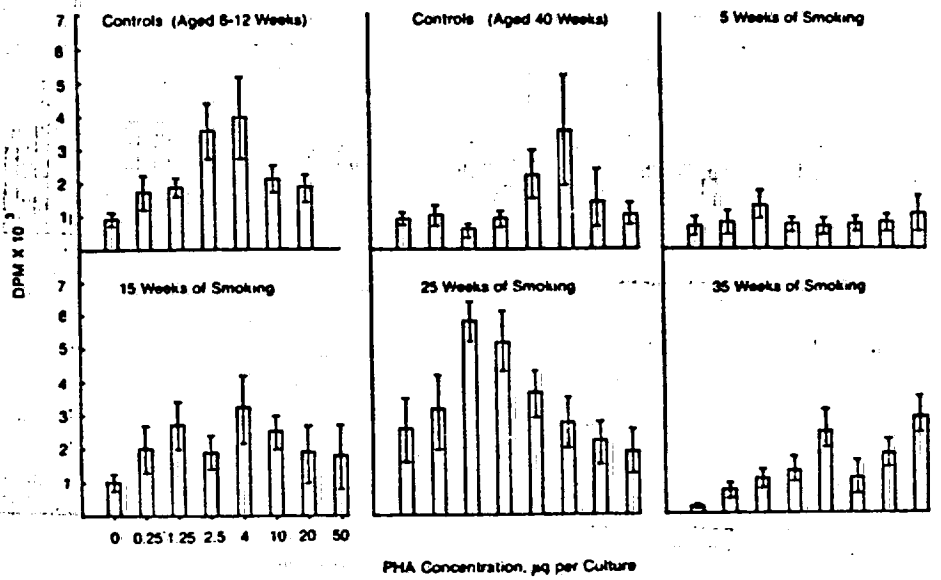

FIGURE 18 The PHA response of splenic lymphocytes. The responses of 6 - 12 week old and 40 week old control mice as well as animals exposed to cigarette smoke for 5, 15, 25 and 35 weeks are shown. Each result represents the mean ± S.E. derived from cultures from three mice. The peak response after 35 weeks smoking was not significantly different from that of control mice as determined by Student's t test.

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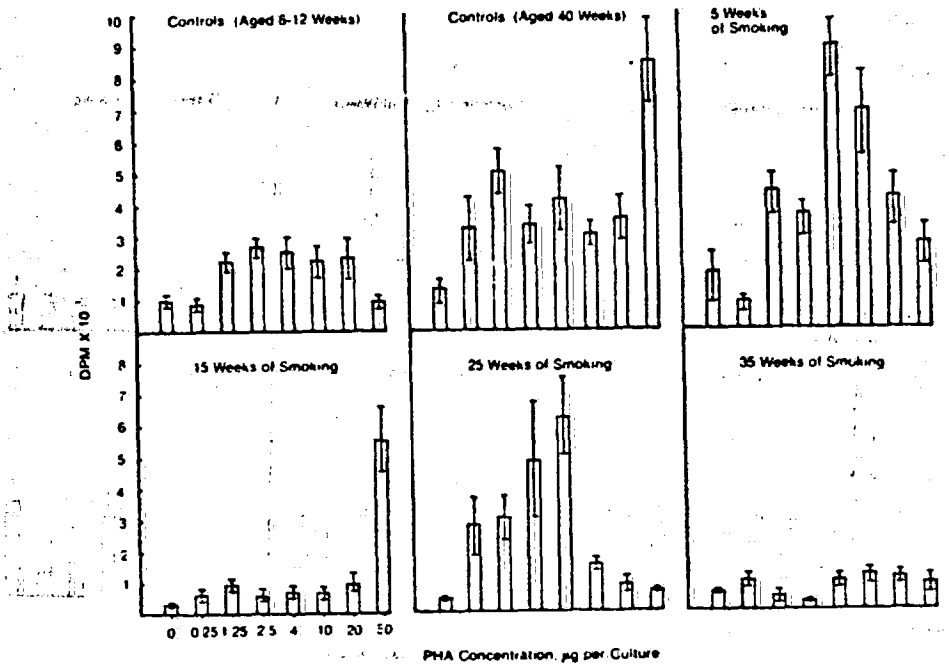

FIGURE 19 The PHA response of cervical and mediastinal lymph node lymphocytes. Each result represents the mean ± S.E. derived from cultures from three mice. The responses of 6 - 12 week old and 40 week old control mice as well as animals exposed to cigarette smoke for 5, 15, 25 and 35 weeks are shown. The peak response after 35 weeks smoking was significantly different from that in control mice as determined by Student's t test ($p<0.01$).

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reactions (World Health Organization 1970; Gowans 1970; Davies *et al* 1971). However as cell-mediated immune reactions can be elicited in the lung without sensitizing the cells in the spleen it appears that the T-cell population throughout the body is not homogeneous (Henney and Waldman 1970). The circulating nature of the PHA-responsive cell is probably demonstrated by the similarity of the changes occurring in the responsiveness of cells of the spleen and peripheral blood to PHA produced by the inhalation of cigarette smoke. The changes in the responses of the lymphocytes from the cervical and mediastinal lymph nodes to PHA, with one major exception, were also similar to the responses of the lymphocytes from the spleen and peripheral blood. After 5 weeks exposure to cigarette smoke the responsiveness of the lymphocytes to PHA from the lymph nodes was enhanced while the responsiveness of the lymphocytes from the blood and spleen was depressed. However, chronic exposure to the cigarette smoke eventually diminished the responsiveness of the lymphocytes in the blood and lymph nodes to PHA. Although the depression of the responsiveness in the spleen was not statistically significant, the tendency of the lymphocytes to respond maximally to higher concentrations of PHA may indicate an impairment. In immunological deficiency syndromes, both the pattern of the PHA dose-response curve and the magnitude of the peak response is altered from normal limits (Hosking, Fitzgerald and Simons 1971).

SECTION 3 PHAGOCYTIC FUNCTION

A. Introduction

The investigations of phagocytic function in mice exposed to cigarette smoke were designed to examine: (i) the ability of the alveolar macrophages to kill bacteria; (ii) the ability of the liver, spleen and lung to remove and process bacteria from the blood.

The phagocytic activity of the alveolar macrophages was determined by measuring the inactivation and clearance of intratracheally inoculated radiolabelled *Pseudomonas aeruginosa*. The inactivation of the bacteria in the lung has been shown to be performed by alveolar macrophages (Green and Kass 1964a,b).

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It has been well established that exposure of macrophages *in vitro* to cigarette smoke diminishes their phagocytic activity (Green and Carolin 1967; Green 1968; Lentz and DiLuzio 1972, 1973). Experiments examining the effect of cigarette smoke exposure on macrophages *in vitro* were performed with emphasis placed on the ability of macrophages exposed to cigarette smoke to regain their phagocytic activity.

B. Pulmonary bacteriocidal activity

Viable, radiolabelled *Pseudomonas aeruginosa* were inoculated intratracheally into female mice which had been exposed to cigarette smoke for 18 or 34 weeks and male mice which were exposed to cigarette smoke for 37 weeks. After 5 and 24 hours the viability and radioactivity of the bacteria inoculated into the lungs of groups of 3 mice were determined. The results (Table 16) are shown as a percentage of values obtained immediately after inoculation. The ratio of the viable bacterial count to the radioactivity is shown as an indication of the viability of the bacteria not cleared from the lungs.

The radioactivity and bacterial counts in the lungs of the female mice exposed to cigarette smoke for 18 weeks were not significantly different from the values of control mice. The female mice exposed to cigarette smoke for 34 weeks had a higher number of viable bacteria remaining in their lungs 24 hours after the inoculation. The male mice exposed to cigarette smoke for 37 weeks had an increased number of viable bacteria remaining in the lung 5 hours after the inoculation. Both the number of viable bacteria and the radioactivity in the lungs of these mice at 24 hours was higher than control mice. The ratio of the bacterial viability and radioactivity in the lung show that the killing of the bacteria not cleared from the lungs was depressed.

C. Blood clearance

Radiolabelled *Pseudomonas aeruginosa* were inoculated intravenously into female mice exposed to cigarette smoke for 18 and 34 weeks and male mice exposed for 37 weeks. The phagocytic indices for their clearance from the blood are shown in Table 17. There was no

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TABLE 16 Pulmonary bacteriocidal and clearance activity of cigarette-smoke-exposed mice

Group[1]		Time[2]	DPM/lung[3]	Bact/lung[4]	Bact/lung / DPM/lung[5]
18 week (female)	Control	0	100.0 ± 13[6]	100.0 ± 8.0[6]	100.0 ± 6.7[6]
	Smoker	5	65.2 ± 6.1	16.4 ± 7.5	28.0 ± 15.0
	Control	5	65.5 ± 15.0	10.0 ± 1.2	16.0 ± 2.3
	Smoker	24	18.4 ± 0.16	0.23 ± 0.057	1.2 ± 0.57
	Control	24	18.7 ± 5.5	0.05 ± 0.0057	0.2 ± 0.04
34 week (female)	Control	0	100.0 ± 22.0	100.0 ± 26.0	100.0 ± 28.0
	Smoker	5	53.0 ± 3.8	8.0 ± 0.57	15.0 ± 1.4
	Control	5	38.0 ± 5.5	8.8 ± 0.63	25.0 ± 0.8
	Smoker	24	12.7 ± 1.0	0.24 ± 0.00057*	2.0 ± 0.17*
	Control	24	13.0 ± 1.0	0.06 ± 0.023	0.4 ± 0.17
37 week (male)	Control	0	100.0 ± 5.8	100.0 ± 13.0	100.0 ± 18.0
	Smoker	5	88.0 ± 5.5	75.0 ± 11.0*	85.0 ± 8.1
	Control	5	61.2 ± 9.4	23.0 ± 4.6	37.0 ± 1.5
	Smoker	24	22.4 ± 1.5*	0.65 ± 0.0034**	4.7 ± 1.1*
	Control	24	11.6 ± 3.1	0.038± 0.0075	0.38 ± 0.17

The significance of the differences between the controls and the smokers was determined by Student's t test: * $p<0.05$, ** $p<0.01$.

(1) Weeks exposure of the smokers to cigarette smoke (sex).

(2) Time after the administration of bacteria to the respiratory tract.

(3) Disintegrations per minute per lung as a percentage of the value obtained immediately after inoculation.

(4) Viable bacteria per lung as a percentage of the value obtained immediately after inoculation.

(5) $\frac{\text{Bacteria/lung (as \% bacteria/lung at time 0)}}{\text{DPM/lung (as \% DPM/lung at time 0)}} \times 10^2$.

(6) Each result is the mean ± S.E. of the values obtained from groups of three mice.

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TABLE 17 Phagocytic indices of the clearance of *Pseudomonas aeruginosa* from the blood of cigarette-smoke-exposed mice

Group	Smoke-exposed	Controls
18 week[1], female	0.075 ± 0.0063[2]	0.075 ± 0.0063
34 week, female	0.089 ± 0.0036	0.089 ± 0.0036
34 week, male	0.050 ± 0.0030	0.051 ± 0.0033

No significant differences between the smoke exposed and control groups were found as determined by Student's t test.

(1) Weeks exposed to fresh cigarette smoke.

(2) Each result is the mean ± S.E. of the phagocytic indices of a group of 6 mice.

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detectable difference between the values of mice exposed to cigarette smoke and corresponding control mice. The radioactivity in the liver, spleen, lungs and kidneys at 10 and 180 minutes after the inoculation of the bacteria is shown in Tables 18 - 20, as a percentage of the inoculum. The radioactivity per gram in each organ, of both mice exposed to cigarette smoke and control mice, was higher than the radioactivity per ml of blood. The mice exposed to cigarette smoke for 34 or 37 weeks had considerably less radioactivity than controls in the liver at 10 minutes and the amount of radiolabel eliminated from the livers between 10 and 180 minutes was also significantly depressed. The amount of radiolabel in the kidneys of mice exposed to cigarette smoke for 18 and 34 weeks was significantly higher than in control mice.

D. Phagocytosis *in vitro*

Cigarette-smoke exposure produced a dose-dependent decrease in the viability and adherence of the macrophages (Fig. 20). The phagocytic activity of the remaining macrophages, as measured over 2 hours, was depressed (Fig. 20). Controls were sham treated. This decrease in phagocytosis was observed if the macrophages were incubated in both high and low concentrations of bacteria (Fig. 21). Phagocytosis was also inhibited when the macrophages were exposed to the vapour phase of cigarette smoke (Table 21). To examine the recovery from the exposure to cigarette smoke the macrophages were exposed to one puff of cigarette smoke and viability and phagocytic activity measured 30 minutes or 24 hours after the smoke exposure. To measure the phagocytic activity of the macrophages in these experiments the macrophages were incubated with bacteria for 2 hours. The experiment was also conducted using macrophages exposed to the vapour phase and macrophages which were exposed to cigarette smoke and immediately washed three times with PBS. The washing was performed by gently filling the petri dishes with 1ml of PBS and immediately removing the PBS. The phagocytic activity in each exposure group was still less than the controls after 24 hours (Table 21). However at 24 hours the macrophages exposed to whole smoke and washed or macrophages only exposed to the vapour phase had a higher phagocytic activity than macrophages exposed to whole smoke.

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TABLE 18 The distribution of radioactivity in female mice exposed to cigarette smoke for 18 weeks after an intravenous inoculation of radiolabelled *Pseudomonas aeruginosa*

Organ	Experimental group	% inoculum per organ[1] 10 mins	180 mins
Liver	Control	30.6 ± 1.8	14.2 ± 0.30
	Smoker	34.3 ± 3.6	16.6 ± 0.86
Lung	Control	3.8 ± 0.32	1.5 ± 0.12
	Smoker	3.5 ± 0.22	1.7 ± 0.18
Spleen	Control	1.1 ± 0.063	1.2 ± 0.075
	Smoker	1.4 ± 0.13	1.6 ± 0.13
Kidney	Control	4.0 ± 0.29	3.8 ± 0.098 **
	Smoker	6.7 ± 0.12 ‡	4.1 ± 0.11
Blood[2]	Control	6.2 ± 0.57	3.1 ± 0.17
	Smoker	6.7 ± 0.46	3.5 ± 0.34

(1) The radioactivity in the organs expressed as a percentage of the amount inoculated was determined 10 and 180 minutes after the inoculation. Each result is the mean ± S.E. of the values from groups of 3 mice.

(2) The percentage of the inoculum in 1 ml of blood.

The significance of the differences between the smoke exposed and control groups was determined by Student's t test: ‡ $p<0.01$.

The significance of the differences in the changes occurring from 10 to 180 minutes between the smoke exposed and controls was determined, as described, by Student's t test: ** $p<0.01$.

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TABLE 19 The distribution of radioactivity in female mice exposed to cigarette smoke for 34 weeks after an intravenous inoculation of radiolabelled *Pseudomonas aeruginosa*

Organ	Experimental group	% inoculum per organ(1) 10 mins	180 mins
Liver	Control	+34.2 ± 1.5	*10.8 ± 1.6
	Smoker	21.6 ± 4.5	13.2 ± 0.40
Lung	Control	1.8 ± 0.18	1.1 ± 0.11
	Smoker	1.6 ± 0.091	1.2 ± 0.057
Spleen	Control	1.2 ± 0.057	0.78 ± 0.14
	Smoker	1.0 ± 0.05	0.76 ± 0.20
Kidney	Control	+4.6 ± 0.30	3.5 ± 0.11
	Smoker	5.7 ± 0.19	3.9 ± 0.18
Blood(2)	Control	3.9 ± 0.44	1.2 ± 0.023
	Smoker	4.0 ± 0.39	0.92 ± 0.063

(1) The radioactivity in the organs expressed as a percentage of the amount inoculated was determined 10 and 180 minutes after the inoculation. Each result is the mean ± S.E. of the values from groups of 3 mice.

(2) The percentage of the inoculum in 1 ml of blood.

The significance of the differences between the smoke exposed and control groups was determined by Student's t test: + $p<0.05$,

The significance of the differences in the changes occurring from 10 to 180 minutes between the smoke exposed and controls was determined, as described, by Student's t test: * $p<0.05$,

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TABLE 20 The distribution of radioactivity in male mice exposed to cigarette smoke for 37 weeks after an intravenous inoculation of radiolabelled *Pseudomonas aeruginosa*

Organ	Experimental group	% inoculum per organ (1) 10 mins	180 mins
Liver	Control	+47.8 ± 2.5	*15.8 ± 2.1
	Smoker	32.2 ± 3.4	15.5 ± 2.0
Lung	Control	3.9 ± 0.33	2.6 ± 0.29
	Smoker	3.6 ± 0.46	2.4 ± 0.35
Spleen	Control	1.5 ± 0.15	2.7 ± 0.35
	Smoker	1.1 ± 0.052	1.9 ± 0.61
Kidney	Control	6.2 ± 0.32	4.5 ± 0.32
	Smoker	6.7 ± 0.80	3.9 ± 0.41
Blood (2)	Control	14.8 ± 0.75	8.8 ± 0.52
	Smoker	13.7 ± 1.6	9.4 ± 1.1

(1) The radioactivity in the organs expressed as a percentage of the amount inoculated was determined 10 and 180 minutes after the inoculation. Each result is the mean ± S.E. of the values from groups of 3 mice.

(2) The percentage of the inoculum in 1 ml of blood.

The significance of the differences between the smoke exposed and control groups was determined by Student's t test: + $p<0.05$.

The significance of the differences in the changes occurring from 10 to 180 minutes between the smoke exposed and controls was determined, as described, by Student's t test: * $p<0.05$.

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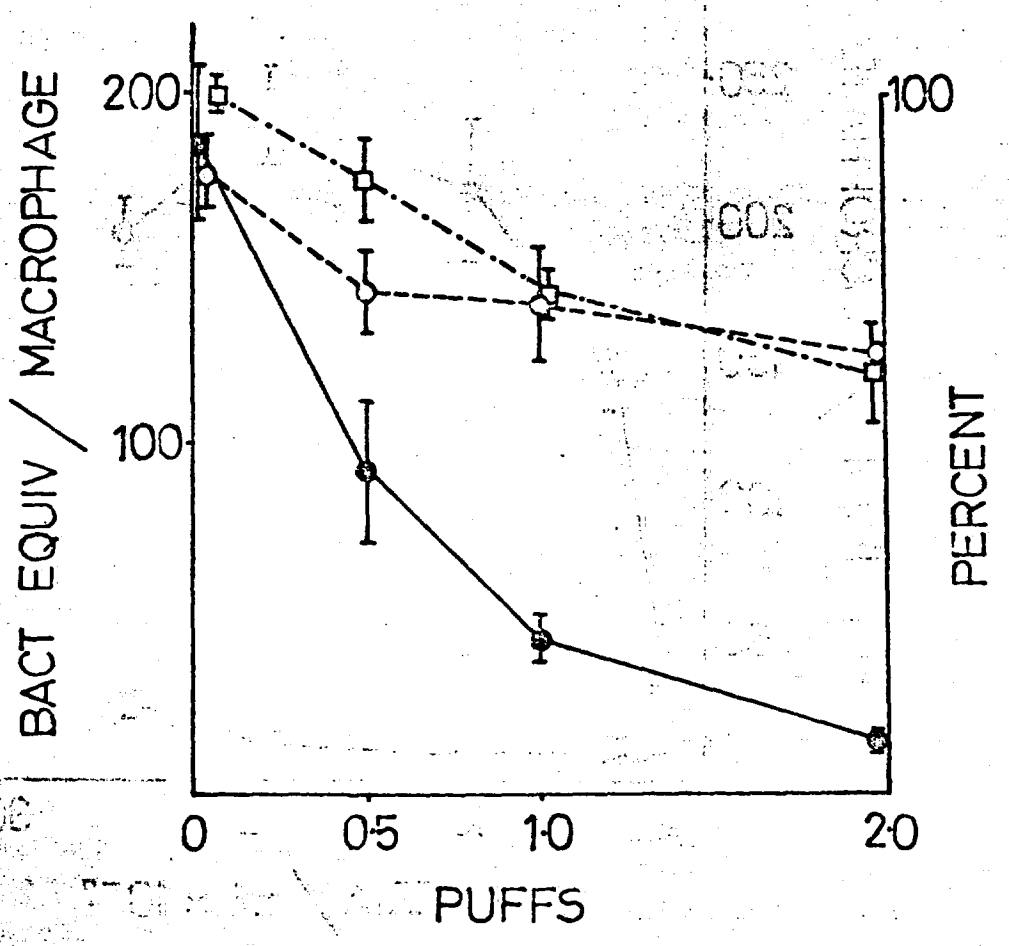

FIGURE 20 Effect of cigarette smoke on viability, adherence and phagocytosis of macrophages *in vitro*. The amount of radioactivity accumulated during 2 hours incubation with radiolabelled *Pseudomonas aeruginosa* was determined as a measure of phagocytic activity. Each result is the mean ± S.E. of the values from 3 cultures.

● phagocytosis, o adherence, □ viability. Viability and adherence (p.34) are shown as percentages of control values and phagocytosis is shown as bact. equivs. phagocytosed.

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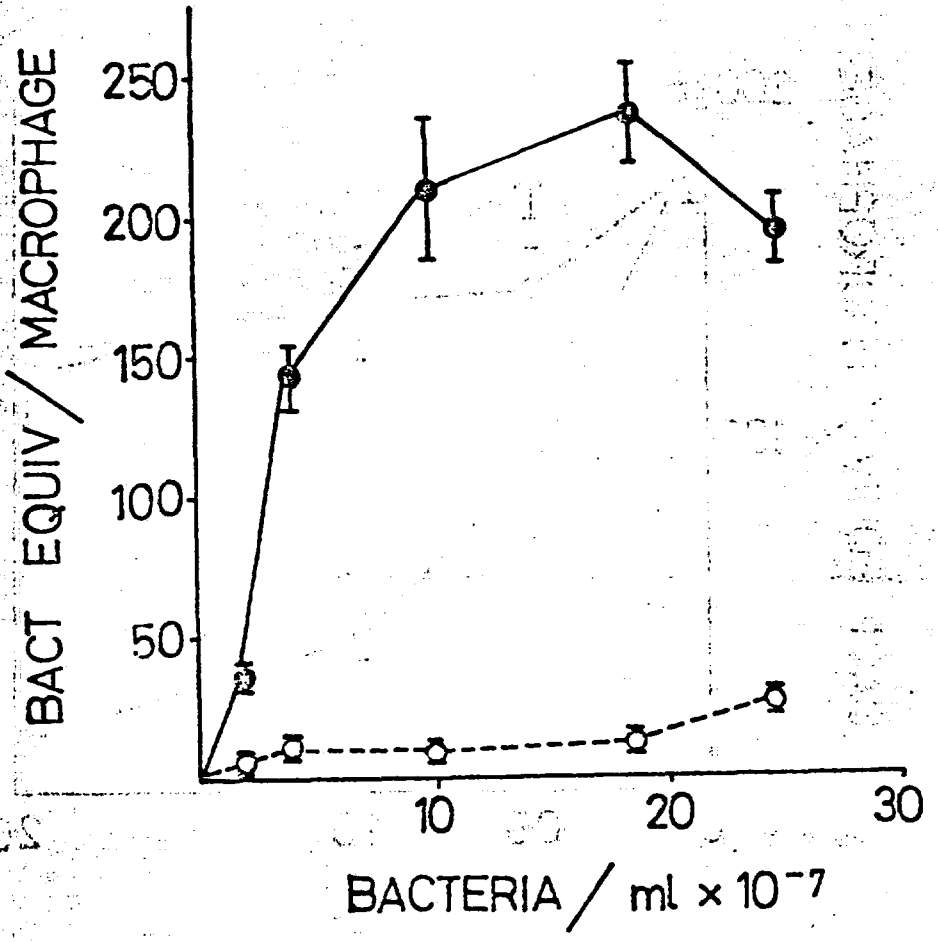

FIGURE 21 Phagocytosis of macrophages exposed to cigarette smoke *in vitro* and incubated for 2 hours in varying concentrations of radiolabelled *Pseudomonas aeruginosa*. ● control, o macrophages exposed to 1 puff of cigarette smoke.

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TABLE 21 The viability and phagocytic activity of macrophages 30 minutes and 24 hours after the exposure to fresh cigarette smoke

	Group	% control(1) 30 minutes	% control(1) 24 hours
Phagocytosis in:	Controls	100.0 ± 2.1	100.0 ± 4.0
	Whole smoke	***21.8 ± 0.96	***28.6 ± 3.5
	Vapour phase	***21.7 ± 3.9	***55.0 ± 5.0
	Whole smoke, (2) washed	***22.9 ± 4.5	***39.7 ± 1.2
Viability	Controls	100.0 ± 3.4	100.0 ± 2.0
	Whole smoke	*56.0 ± 11.0	**50.3 ± 6.7
	Vapour phase	**54.4 ± 11.0	*74.7 ± 7.4
	Whole smoke, washed	*73.3 ± 8.4	***54.0 ± 1.5

(1) The results are expressed as a percentage of the corresponding control value. Each result is the mean ± S.E. of three cultures.

(2) Immediately after smoke exposure the macrophages were washed with three 2 ml changes of PBS.

The significance of the differences between the tests and the controls were determined by Student's t test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

After 24 hours the phagocytosis/macrophage by macrophages exposed to vapour phase or whole smoke and washed was significantly different from cells only exposed to whole smoke ($p<0.01$).

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When the radioactivity accumulated by macrophages phagocytosing for periods up to 5 hours was measured, it was found that one puff of the cigarette smoke caused a depression of radioactivity detected after 2 hours but in contrast increased the radioactivity accumulated in 5 hours (Fig. 22). To examine the possibility that the increased accumulation of radioactivity produced by exposure to cigarette smoke could be caused by a defect in the ability of macrophages to degrade and eliminate bacteria, the ability of the macrophages to eliminate phagocytosed bacteria was measured. Macrophages were incubated with bacteria for 3 hours and an enhanced accumulation of radioactivity occurred in the macrophages exposed to cigarette smoke. The elimination of the radioactivity measured over the next three hours was faster than control macrophages (Table 22). The vapour phase of cigarette smoke could also stimulate the accumulation of radioactivity (Table 23). In subsequent experiments it was found that different preparations of the radiolabelled *Pseudomonas aeruginosa* influenced the effect of cigarette smoke. Although it was attempted to standardize the preparations of different batches of bacteria, when some preparations were used the stimulation could be detected after 2 hours of phagocytosis while with others the stimulation could not be detected at 5 hours.

E. Discussion

Previous data has shown that the mice used in these experiments have a larger number of alveolar macrophages (Holt and Keast 1973a) than control mice. The finding that bacteriocidal activity in their lungs was depressed despite the increase in alveolar macrophages concurs with results obtained by Rylander using guinea pigs (Rylander 1972a,b). These results indicate that the bacteriocidal activity of macrophages in the lung was depressed by the inhalation of cigarette smoke. The clearance of the radiolabelled bacteria from the lungs of male mice exposed to cigarette smoke was also found to be depressed.

Previous reports have described the inhibitory effect of cigarette smoke and its vapour phase on the phagocytic activity of macrophages cultivated *in vitro* (Green and Carolin 1967; Green 1968; Lentz and DiLuzio 1972, 1973). The experiments with macrophages

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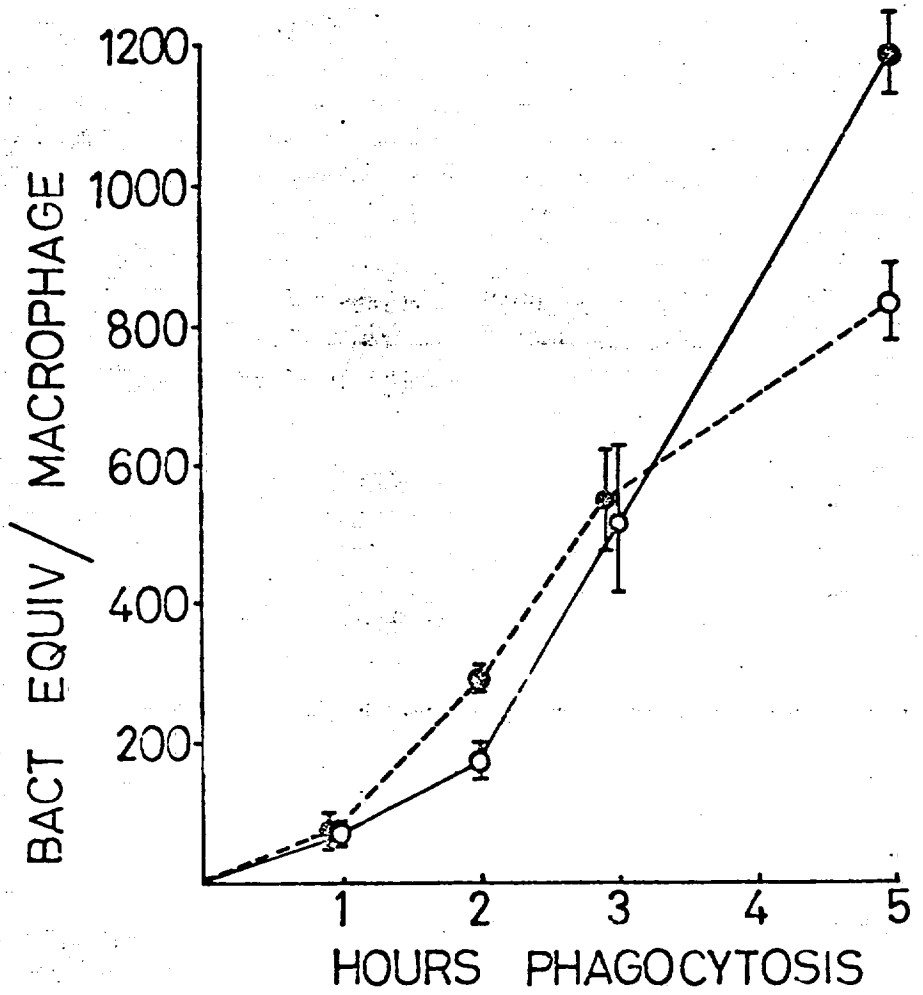

FIGURE 22 Effect of exposure to cigarette smoke on the accumulation of radioactivity from radiolabelled *Pseudomonas aeruginosa* by macrophages *in vitro*. ● control, o macrophages exposed to 1 puff of cigarette smoke. The significance of the difference between control and smoke exposed macrophages was determined by Student's t test. The radioactivity in the test and control macrophages was significantly different at 2 hours ($p<0.02$) and 5 hours ($p<0.01$).

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TABLE 22 The elimination of phagocytosed radiolabelled *Pseudomonas aeruginosa* from cigarette-smoke-exposed macrophages

	Phagocytosis	Elimination	
	(Bact. equiv/cell)	(Bact. equiv/cell)	%(1)
Control	246 ± 40	119	48
Smoke exposed	700 ± 142	280	40

After exposing the macrophages to 1 puff of cigarette smoke the phagocytosis after three hours and the elimination after a further three hours were measured. Triplicate cultures were used (mean ± S.E.)

(1) Percent of the radiolabel phagocytosed.

TABLE 23 The effect of the vapour phase on the accumulation of radiolabelled bacteria by macrophages *in vitro*

Time of phagocytosis	Exposure	
	Control	Vapour phase
2 hours	112 ± 16*	75 ± 3
5 hours	267 ± 23	413 ± 33

* Bacteria equivalents of radiolabel/macrophage.

Each result represents the mean ± S.E. of the values obtained from 3 cultures.

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in vitro described here have used peritoneal macrophages as previous data has shown that cigarette smoke produces similar changes in the RNA and protein synthesis of alveolar and peritoneal macrophages (Holt and Keast 1973b,d). When the macrophages began to phagocytose bacteria after exposure to cigarette smoke their rate of accumulation of radioactivity was depressed, but they were eventually able to accumulate radioactivity more rapidly than control macrophages. This phenomena did not appear to be caused by a defect in the ability of the macrophages to digest and eliminate the radioactivity. Metabolically inactive macrophages are more susceptible to toxic effects of cigarette smoke (Holt and Keast 1973b). It is possible that macrophages with a low phagocytic rate could be killed by the cigarette smoke leaving a population with a higher average phagocytic rate. This was not the only factor as the total phagocytosis of a monolayer of macrophages exposed to cigarette smoke often exceeded the total phagocytosis of a control monolayer. The vapour phase could also produce both the stimulation and inhibition of phagocytosis. It is not known how the different preparations of *Pseudomonas aeruginosa* slightly influenced the results as it was attempted to standardize the preparation of bacteria.

The results in these experiments showed that the macrophages accumulated large amounts of bacteria. This was consistent with morphological observations and the fact that continued phagocytosis was toxic for the macrophages (Thomas, Holt and Keast 1974b). Without allowing for digestion of the phagocytosed bacteria, the volume of bacteria accumulated by the macrophages reached about 20% of the volume of the macrophages. The stimulatory effect of endotoxins from the bacteria (Stuart 1970) may account for large amounts of phagocytosis. It is also possible that the digestion of bacteria within macrophages decreased the self-absorption of radioactivity by the bacteria and this exaggerated the measurement of phagocytosis.

Macrophages incubated without bacteria recovered slowly from the inhibitory effect of cigarette smoke on their phagocytic activity. The difference in the rate of recovery of the macrophages exposed to whole smoke and vapour phase indicated that more than one component in cigarette smoke inhibits phagocytosis.

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In experiments reported by Green (1968), macrophages cultured in medium containing fresh serum, inactivated 70% of bacteria added to the cultures, while macrophages incubated in medium without serum only inactivated 43% of the bacteria. However macrophages which had been exposed to cigarette smoke could inactivate 49% of the bacteria in the absence of serum, but only 1% of the bacteria in the presence of serum. The absence of serum may correspond to the heat-treated serum used for the experiments described in this section. It is apparent that the direct effect of cigarette smoke on macrophages is complex, but when evaluating the effect of cigarette smoke on phagocytosis it is important to consider that relatively small doses of cigarette smoke were highly toxic for macrophages and caused considerable cell death.

The clearance of radiolabelled bacteria from the blood, as measured by the phagocytic index, was unaffected in mice chronically exposed to cigarette smoke. However 10 minutes after the inoculation of bacteria the livers of mice chronically exposed to cigarette smoke contained considerably less radioactivity than the livers of control mice. The decrease of radioactivity in the liver from 10 to 180 minutes was greatest in the control animals. Other interpretations of these results are possible, but the results strongly suggest that the phagocytic and degradative activity of the liver phagocytes was impaired by the inhalation of cigarette smoke. The most obvious source of radioactivity in the kidney was material phagocytosed, degraded and eliminated from phagocytic cells. This radioactivity was increased in mice exposed to the cigarette smoke, even when the radioactivity in the liver was the same as the control. This would mean that another phagocytic component with a rapid rate of digestion of the bacteria was operating in mice exposed to cigarette smoke. One possibility is the circulating polymorphonuclear leucocytes. A decrease in liver function may mean that its important detoxifying action on endotoxins in the portal blood is decreased (Thomas, McSween and White 1973). This could have important effects on the immune system and the health of the animal, as will be discussed in Chapter 5.

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SECTION 4 GROWTH OF TRANSPLANTED TUMOURS

A. Introduction

It was considered that if immunosuppression, produced by the inhalation of cigarette smoke, could allow neoplasms to escape from immunological control (Holt, Thomas and Keast 1973; Keast and Holt 1974), it should be possible to demonstrate that growth of established immunogenic neoplasms in mice is enhanced by cigarette-smoke inhalation. Two experimental systems were used to examine this possibility: (i) the ability of the Lewis lung tumour to grow subcutaneously and mestastasise to lungs, (ii) the growth of intra-tracheally-inoculated TKL5 tumour cells.

The Lewis lung tumour originated as a spontaneous lung carcinoma in a C57Black mouse and was routinely maintained by serial passage in adult mice. Immunostimulants have been shown to inhibit the growth of this tumour (Renoux and Renoux 1972). The TKL5 tumour cells were from a tumour induced by a murine sarcoma virus (see Chapter 2 section M) and were maintained in tissue culture. These cells did not readily grow in adult mice. The antigenicity of tumours induced by murine sarcoma viruses is well documented (Fefer, McCoy and Glynn 1967; Law, Ting and Stanton 1968).

B. Subcutaneous growth and metastasis of Lewis lung tumour

C57Black mice were exposed to fresh cigarette smoke for 3 days, 23 weeks and 38 weeks prior to the subcutaneous inoculation of 10^6 Lewis lung tumour cells. Cigarette-smoke exposure was continued and the tumour growth was estimated by measuring diameters. The growth of tumours in mice exposed to cigarette smoke for three days before the inoculation was almost identical to the growth of tumours in control mice (Fig. 23). However the growth of the tumours in mice that had been exposed to cigarette smoke for 23 and 38 weeks was significantly increased as compared to control animals (Fig. 23). The lungs were examined for metastases. The mice exposed to cigarette smoke for 23 and 38 weeks had 2.8 ± 1.1 (Mean ± S.E.) while age-matched control mice had 0.8 ± 0.4 metastases per lung.

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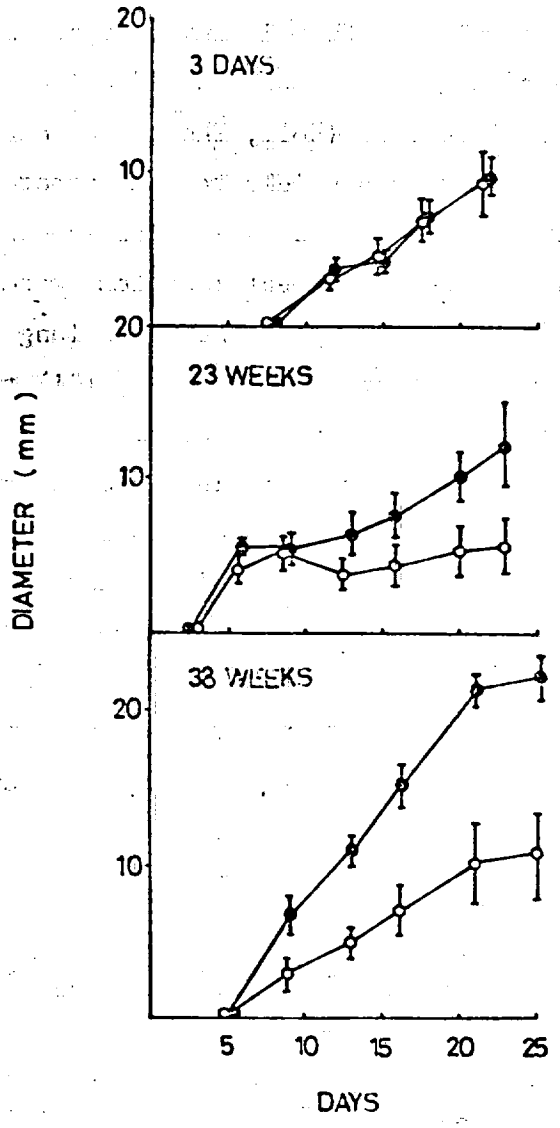

FIGURE 23 The subcutaneous growth of the Lewis lung tumour in mice exposed to cigarette smoke for 3 days, 23 weeks and 38 weeks. Each result is the mean ± S.E. of the tumour diameter of 10 mice. Smoke exposed ●, Control o. As determined by Student's t test there was no significant difference between the tumour growth in mice exposed to cigarette smoke for 3 days and controls but the diameter of the tumours in mice exposed to cigarette smoke for 23 and 38 weeks became significantly different from their corresponding controls 20 and 9 days after the tumour inoculation, respectively.

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C. Growth of intratracheally-inoculated tumour cells

BALB/c mice were exposed to cigarette smoke for 20 weeks and then inoculated intratracheally with 10^5 TKL5 tumour cells. As shown in Table 24 (Exp. 1), 6/13 of the smoke-exposed mice died or became moribund. None of the control mice died within 50 days. In another experiment (Table 24 Exp. 2) a group of BALB/c mice were exposed to cigarette smoke for 31 weeks before receiving the intratracheal inoculation of TKL5 cells. As well as a group of control mice, which were not exposed to cigarette smoke, this experiment also included a group of mice, the same age, but only exposed to cigarette smoke on the 3 days preceding the inoculation. Only mice exposed to cigarette smoke for 31 weeks died or became moribund (6/15). The lungs of mice which died after the inoculation of tumour cells were examined histologically. They exhibited numerous foci of neoplastic proliferations varying in size from a few cells to large extensive zones of neoplastic growth (Fig. 24) which completely replaced the whole lobes of the lungs. Generally the foci were small, consisting of 50 - 200 cells and were situated near bronchi or small vessels. Mitoses were present. In the cases where foci were large, vasoproliferation became evident together with an infiltrate of lymphocytes, macrophages and giant cells.

D. Discussion

The results show that the subcutaneous growth of the transplantable Lewis lung tumour was significantly increased in mice chronically exposed to cigarette smoke. Mice chronically exposed to cigarette smoke showed a higher number of metastases in the lung than control mice, but this difference was not statistically significant. However the TKL5 tumour cells only grew significantly in the lungs of mice chronically exposed to cigarette smoke. The experiments measuring the clearance of intratracheally-inoculated bacteria (Chapter 4 section 3.B) indicate that depression in tracheobronchial-clearance activity was not responsible for increasing the susceptibility of mice exposed to cigarette smoke to intratracheally - inoculated tumour cells.

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TABLE 24 The response of cigarette-smoke-exposed mice to an intratracheal inoculation of TKL5 tumour cells

	Smoke Exposure	No. dead* / No. inoculated	Days to death Mean (range)
Exp. 1	20 weeks	6/13	28.6 (14-40)
	Control	0/19	
Exp. 2	31 weeks	6/15	15.0 (10-46)
	3 days	0/20	
	Control	0/20	

* Number of mice dead or moribund.

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The experiments indicate that inhalation of cigarette smoke was able to modify conditions within the smoking animal that result in the promotion of growth of an established malignant cell. The fact that conditions for the production of enhanced tumour growth did not occur after short term exposure to cigarette smoke (3 days) indicated that either an accumulation of substances acting directly on tumour cells must occur or that cigarette-smoke inhalation eventually impairs a mechanism controlling tumour growth. These results are consistant with the concept that immunosuppression, induced by the inhalation of cigarette smoke, allows immunogenic neoplastic cells to escape from immune control.

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CHAPTER 5

GENERAL DISCUSSION

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FIGURE 24 Growth of intratracheally-inoculated TKL5 cells in the lungs of mice exposed to cigarette smoke.

TOP: Aggregates of large malignant cells are found in the near vicinity of an arteriole and a small bronchiole. A mononuclear infiltrate surrounds the aggregates. Magnification 140 X.

BOTTOM: A large neoplastic mass has completely replaced much of the parenchyma of the lung. Magnification 87 X.

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The Second World Conference on Smoking and Health concluded that there was a need to develop experimental models to assist in producing less harmful cigarettes and determining how cigarette smoking produces disease (Workshop of the Second World Conference on Smoking and Health 1972). An understanding of the mechanism(s) by which cigarette smoking produces disease could be useful in identifying cigarette smokers with a high risk of developing disease, in the treatment and prophylaxis of the diseases associated with cigarette smoking and for developing bioassay systems for evaluating the safety of tobacco products and substitutes. The mechanism(s) of action of cigarette smoke on experimental models should relate to the mechanism(s) of producing disease in humans.

It has also become apparent, because of the aging of cigarette-smoke components (Hoffmann and Wynder 1970), the effects of components of the vapour phase of cigarette smoke (Leuchtenberger and Leuchtenberger 1970a) and the method used by humans to administer cigarette smoke to themselves, that the experimental models should involve the inhalation of fresh cigarette smoke (Dontenwill 1970; Hoffmann and Wynder 1970; Leuchtenberger and Leuchtenberger 1970). Active inhalation experiments have been performed where animals inhale air through a burning cigarette (Albert *et al* 1969; Auerbach *et al* 1970a), but these techniques require large animals and difficulties are encountered when experiments require the use of large numbers of animals (Hoffmann and Wynder 1970). Hence inhalation chambers, like the Hamburg II smoking apparatus, have been developed in order to expose laboratory rodents to cigarette smoke. The development of animal models for studying biological effects of cigarette smoking, as well as attempting to simulate the smoking habit, also represent attempts to reproduce disease processes which may take 20 - 30 years to develop in human smokers (Royal College of Physicians 1971; United States Public Health Service 1971).

An examination of the immune system of animals exposed to cigarette smoke, as well as the immune system of human smokers may be important in determining both the suitability of the animal as a model for studying diseases associated with cigarette smoking and the role of immune dysfunction, if any, in the aetiology of these diseases. The influence of cigarette smoking on immunity is

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pertinent because immunosuppression may, at least in part, contribute to the aetiology of the elevated incidence of infection and neoplasia associated with cigarette smoking (Chapter 1 section A). While the relevance of immunological changes, produced in animals by the inhalation of cigarette smoke, to human disease can only be determined after investigations into the immune system of human smokers, results of studies with inbred animals can be used as a guide for designing studies of human smokers.

In the experiments described in Chapters 2, 3 and 4, inbred mice were exposed to cigarette smoke in a Hamburg II smoking apparatus (Dontenwill 1970) for 7 minutes on five days of each week for periods up to 42 weeks. Although the inhalation of cigarette smoke produced some depression in the appetite, body weight and haemoglobin concentration in the mice the changes were not large and the mice remained apparently healthy during the experiments (Chapter 2).

The primary humoral immune responses of mice to SRBC, an immunogen requiring T-cells, (Davies *et al* 1971; Playfair 1971), were transiently enhanced by the inhalation of cigarette smoke but after chronic inhalation of smoke these immune responses were markedly depressed (Chapter 4 sections 1.C and 1.D; Thomas, Holt and Keast 1973a, 1974c). The depressions were evident when SRBC were inoculated intraperitoneally or intratracheally, and were shown by decreases in the antibody-forming cell response in the cervical and mediastinal lymph nodes, lungs and spleen as well as serum antibody titres. These changes were at least partially reversible by discontinuing smoking (Chapter 4 section 1.C; Thomas, Holt and Keast 1974d). However serum antibody responses to PVP, an immunogen not requiring T-cells (Andersson and Blomgren 1971), were not enhanced or depressed by inhalation of cigarette smoke (Chapter 4 section 1.F). This indicates that cigarette smoke had little direct effect on the antibody-producing cells and antibody in mice, a supposition supported by experiments with antibody and antibody-forming cells *in vitro* (Chapter 4 section 1.G), and the finding that short term inhalation of cigarette smoke did not decrease the humoral immune responses to SRBC (Chapter 4 section 1.C and 1.D; Thomas, Holt and Keast 1974c).

The transient stimulation of the humoral immune response to SRBC by cigarette smoke did not appear to be mediated by infection or exposures to an agent antigenically related to SRBC as the number

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of antibody-forming cells in the spleen and lymph nodes of mice which were unimmunized but exposed to cigarette smoke was the same as in unimmunized control mice (Chapter 4 section 1.E). Esber *et al* (1973) did not show a transient enhancement in the humoral immune response of mice, exposed to cigarette smoke, to SRBC. Instead, after only five days exposure to the cigarette smoke, mice had severely depressed primary and secondary haemagglutinating antibody responses to intraperitoneal inoculations of SRBC. However the regime of exposure to cigarette smoke used by Esber *et al* (1973) was considerably more severe than the exposures used in the present experiments (Chapter 3).

The ability of mice, chronically exposed to cigarette smoke to effect cell-mediated immune reactions, as judged by the responsiveness of their lymphocytes to PHA, was impaired (Chapter 4 section 2.B; Thomas, Holt and Keast 1973b). However the responsiveness of lymphocytes from cervical and mediastinal lymph nodes to PHA was transiently enhanced by the inhalation of cigarette smoke, similar to the immune responses to SRBC. These reactions have also been shown to be accompanied by a transient infiltration of lymphocytes into the lungs of mice (Holt and Keast 1973a). The similarities in the changes occurring in the responsiveness of lymphocytes from mice exposed to cigarette smoke to PHA and antibody responses to an immunogen requiring T-cells, SRBC, suggest that inhalation of cigarette smoke produces a defect in T-cell function. Experiments showing that antibody responses, to PVP, an immunogen not requiring T-cells, were not influenced by inhalation of cigarette smoke, support this concept.

When macrophages were exposed to cigarette smoke *in vitro* and then incubated with bacteria their initial phagocytic activity was depressed, but if the incubation with bacteria was continued the phagocytic activity of the macrophages was higher than the phagocytic activity of control macrophages (Chapter 4 section 3.D). The ultimate effect of inhalation of cigarette smoke on the function of alveolar macrophages was estimated by determining the bacteriocidal activity in the lungs of mice (Chapter 4 section 3.B). Pulmonary bacteriocidal activity, which has been attributed to the activity of alveolar macrophages (Green and Kass 1964a,b), was depressed in mice chronically exposed to cigarette smoke. This depression occurred

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despite the fact that mice exposed to cigarette smoke had increased numbers of alveolar macrophages (Holt and Keast 1973a). Experiments conducted by Rylander (1971a,b) similarly showed that guinea pigs, exposed to cigarette smoke, had depressed pulmonary bacteriocidal activities in the presence of elevated numbers of alveolar macrophages. Human cigarette smokers have also been shown to have a higher number of alveolar macrophages than nonsmokers. However when alveolar macrophages were cultivated *in vitro*, their phagocytic activity was similar to that of alveolar macrophages cultivated from nonsmokers (Cohen and Cline 1971; Harris *et al* 1971; Mann *et al* 1971). It is possible that the measurement of phagocytic capacity of alveolar macrophages *in vitro* may not be a sufficient measure of bacteriocidal activity in the lungs. Alterations in phagocytic cells could also account for other changes in the immune response produced by cigarette smoke inhalation. The influence of macrophages on the antibody response to SRBC is well documented (Ford, Gowans and McCullough 1967; Mosier 1967; Shortman *et al* 1970; Cruchard and Unanue 1971).

The function of liver phagocytes was also apparently depressed in the mice chronically exposed to cigarette smoke (Chapter 4 section 3.L). This impairment could decrease the removal of endotoxins from portal blood (Thomas, McSween and White 1973; Woodruff, O'Carroll, Koizumi and Fine 1973), which have been shown to be capable of either enhancing or inhibiting the immune response (Franzl and McMaster 1968; Neter 1969; Chedid 1973; Jutila 1973). Laurenzi, Gaurneri and Endriga (1965) have shown that either enteric bacteria or an intraperitoneal inoculation of endotoxin was required to allow acute exposures of cigarette smoke to inhibit the removal of viable bacteria from mouse lungs. There is also evidence suggesting that endotoxins can weaken the immunosurveillance mechanisms against neoplasms (Stanley and Keast 1967; Keast 1968; Keast, Stanley and Phillips 1968; Keast and Stanley 1969). However no leucocytosis was found in the mice as might be expected from the actions of endotoxin (Jutila 1973). Substances found in cigarette smoke, carbon particles and cadmium (Lewis, Jusko, Coughlin and Hartz 1972), have also been shown to be capable of both enhancing or inhibiting immune responses (Jones, Williams and Jones 1971; Zarkower 1972a,b).

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The exposure to cigarette smoke involves forcing the mice to inhale an irritant. While the physical handling of the mice has been found not to influence humoral immune responses (Esber *et al* 1973) or body weights (Leuchtenberger and Leuchtenberger 1970a) it is difficult to evaluate the effect of the stress produced by compelling the mice to inhale the smoke. The problem has been approached by examining temporal aspects of the alterations produced in the immune system. In particular it was thought that changes occurring soon after commencing cigarette smoke exposure were likely to be due to stress; during this time the mice displayed movements indicative of discomfort. Except for the pfc response in the lungs of mice after intratracheal inoculations of SRBC, no depressions in the immune response were found before 20 weeks of exposure to cigarette smoke, however some immune responses were enhanced (Chapter 4 section 1.C and 1.D). It appears improbable that the depressions in immune responses ensuing in the following 20 weeks should be due to stress, especially when the mice had become accustomed to smoke exposure. A similar sequence of transient enhancement and then depression in immune responses has been noted in mice exposed to carbon and oxidant gas (Zarkower 1972a,b).

Although primary immune responses of mice exposed to cigarette smoke to SRBC were depressed, it was apparent that mice could mount large immune responses to continued antigenic challenge. This poses the question whether or not the immunodeficiencies noted in mice exposed to cigarette smoke can influence the resistance of mice to neoplasia and infection. Similarly the phagocytic activity in the lungs of mice exposed to cigarette smoke, although less efficient than control mice, was effective in inactivating large numbers of bacteria (Chapter 4 section 3.B). The results of experiments described in this thesis (Chapter 4 section 4.B and 4.C) showed that the growth of established malignant cells was increased in mice exposed to cigarette smoke, when compared to their growth in age-matched-control mice. Spurgash *et al* (1968) have shown that mice exposed to cigarette smoke are also more susceptible to respiratory infection. Studies on the influence of inhalation of cigarette smoke on pulmonary tumorigenesis in mice exhibiting a relatively high natural incidence of lung adenomas and adenocarcinomas, showed an earlier onset and increased incidence of these neoplasms (Leuchtenberger and Leuchtenberger 1970a; Leuchtenberger, Leuchtenberger and Rossier

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1973). However, when a strain of mice with a lower natural incidence of these neoplasms was examined, under the same experimental conditions, no increase in the incidence of neoplasms was found (Leuchtenberger, Leuchtenberger and Rossier 1973). These studies are consistant with proposals that inhalation of cigarette smoke produces a significant depression in the ability of the immune system of mice to control neoplastic growth and infection.

There are also indications that cigarette smoking impairs the ability of the human immune system to control infection and neoplasia. Cigarette smokers have been found to have a less persistant serum antibody response to influenza virus infection and vaccination than nonsmokers (Finklea, Hasselblad, Riggan, Nelson, Hammer and Newill 1971). Alveolar macrophages from cigarette smokers, but not from nonsmokers, have been reported to be refractile to MIF (Macrophage Migration Inhibition Factor), a product produced by activated lymphocytes, which probably has an important function in recruiting macrophages into cell-mediated immune reactions (Warr and Martin 1973b). Cigarette smokers have a higher number of alveolar macrophages than nonsmokers (Harris *et al* 1970a), similar to the increased number of alveolar macrophages found in mice and guinea pigs exposed to cigarette smoke (Rylander 1971a; Holt and Keast 1973a), but when alveolar macrophages from smokers were cultivated *in vitro* their phagocytic activity was similar to that of macrophages from nonsmokers (Harris *et al* 1971a; Mann *et al* 1971; Cohen and Cline 1971). As both mice and guinea pigs exposed to cigarette smoke have decreased pulmonary bacteriocidal activities, despite elevated numbers of alveolar macrophages, it is possible that cigarette smokers, who also have elevated numbers of alveolar macrophages, have depressed pulmonary bacteriocidal activity and that measurement of phagocytic activity *in vitro* does not reflect phagocytic activity *in vivo*. A discrepancy exists between the effect of cigarette smoke on the peripheral blood leucocytes of mice and humans. Cigarette smokers have a higher number of peripheral blood leucocytes than nonsmokers (Howell 1970; Corre *et al* 1971; Friedman *et al* 1973) but exposure to cigarette smoke decreases the number of peripheral blood leucocytes in mice (Chapter 3 section F). Until the role of infection in elevating leucocyte counts of human smokers is determined (Friedman *et al* 1973) these differences cannot be fully evaluated.

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Although the immunosuppressive effect of cigarette smoke may easily be considered to account for the increased respiratory infection of cigarette smokers, the participation of immunosuppression, if any, in the aetiology of the human lung cancer attributed to cigarette smoking may be complex. Mice chronically exposed to cigarette smoke have an increased incidence of adenocarcinoma (Harris and Negroni 1967; Leuchtenberger and Leuchtenberger 1970a), which is the common pulmonary neoplasm of mice (Stewart, Dunn and Snell 1970). Adenocarcinomas are also the most frequent human lung cancer (Berg 1970), but these neoplasms constitute only a small proportion of lung cancer attributed to the effects of cigarette smoking (United States Public Health Service 1971; Royal College of Physicians 1971). Furthermore, filters have been shown to increase the carcinogenic activity of cigarette smoke in mice (Leuchtenberger and Leuchtenberger 1970) but decrease the incidence of lung cancer in humans (Royal College of Physicians 1970; United States Public Health Service 1971). These findings support proposals implicating an immunosuppressive effect, of a substance in the vapour phase of cigarette smoke, in producing the increased incidence of lung cancer in mice and the action of carcinogens in the particulate phase of cigarette smoke in producing human lung cancer. However the role of carcinogens in cigarette smoke in initiating human lung cancer is not clear. The high incidence of lung cancer in uranium and asbestos workers appears to be dependent on cigarette smoking but the occupational contaminants appear, at least in part, to determine the histology of the cancers (Selikoff, Hammond and Churg 1968; Churg and Kannerstein 1970; Archer *et al* 1973). There is also some evidence indicating that common atmospheric pollutants may initiate a proportion of lung cancers which are then promoted by cigarette smoking (Cannow and Meier 1973). While it is well documented that cigarette smoke contains substances which can initiate and promote carcinogenesis (Wynder and Hoffmann 1964; Stedman 1967; United States Public Health Service 1971), there may be many instances when carcinogenesis is initiated by an agent other than cigarette smoke but the tumour promoting activity of cigarette smoke is required for the development of lung cancer. Immunosuppression, induced by cigarette smoking, may be responsible for promoting carcinogenesis

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induced by carcinogens from cigarette smoke or other sources.

There are also several possible inter-relationships between infection, immunosuppression and carcinogenesis which may be relevant to the diseases found in cigarette smokers. Respiratory tract infection can decrease the bacteriocidal activity in the lungs (Green 1965; Klein *et al* 1968; Jakab and Green 1973). This would provide a mechanism to amplify the immunosuppression induced by exposure to cigarette smoke. Viruses could exert a cocarcinogenic effect by changing the metabolism of cells to render them more susceptible to carcinogens or by producing immunosuppression; the infection of animals with virus has been shown to increase their susceptibility to chemical carcinogens (Kotin and Wisely 1963; Southam *et al* 1969; Nettesheim, Richters and Liziby 1972). However virus infection has also been shown to decrease the adenomas and adenocarcinomas produced in X-radiated mice with chemical carcinogens (Nettesheim *et al* 1970). The incidence of atypical bronchial proliferations produced by cigarette smoke has also been found to be increased by influenza infection (Leuchtenberger *et al* 1963). The role of the metaplastic changes in the production of cancer is not known (Saccomanno *et al* 1970) but chronic infection with parainfluenza virus or mycoplasma can produce metaplastic lesions resembling squamous cell carcinomas (Richter 1970).

A depressed immune system could also facilitate the activity of oncogenic viruses. The interaction of oncogenic viruses and cigarette smoke has been considered (Leuchtenberger, Leuchtenberger, Zebrun and Schaffer 1960; Leuchtenberger 1970). Condensate from cigarette smoke has been shown to induce C-type oncogenic viruses from cells *in vitro* (Freeman *et al* 1971) and infection with C-type virus has been shown to render cells more susceptible to transformation with chemical carcinogens (Rhim *et al* 1972; Saleno, Ramm and Whitmire 1973).

As outlined above, several factors may be involved in the aetiology of lung cancer in cigarette smokers. Immunosuppression may contribute to both the induction and promotion of lung cancer in cigarette smokers and animals exposed to cigarette smoke, as well as respiratory infection. Experiments with animals and investigations of cigarette smokers have shown that cigarette smoke can damage immune function.

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